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UNVEILING POPULATION DIVERSITY, BIOSURFACTANTS AND ANTIBACTERIAL AGENTS PRODUCTION OF *BACILLUS PUMILUS* GROUP SPECIES

Tese do 3º Ciclo de Estudos conducente ao Grau de Doutoramento em Ciências Farmacêuticas na especialidade de Microbiologia

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Aos meus pais e madrinha

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> ***Somewhere, something incredible is waiting to be known.***
>
> *(Carl Sagan)*

Abstract

Nature and its huge biodiversity harbor an endless source of compounds containing unique chemical structures with interesting antimicrobial activity. Particularly, those from microorganisms have played an important role in the discovery of therapeutic agents for infectious diseases.

The recent rise in multidrug resistance (MDR) bacteria, and particularly Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant Enterococci (VRE), has become a major concern due to the increased morbidity and mortality, presenting a negative economic and social impact. Therefore, it is urgently needed to identify and develop new antibacterial compounds or alternative anti-infectious strategies.

During several decades, in some regions of Portugal, a slug was used as traditional medicine in the treatment of cutaneous infections. In a previous work we demonstrated a strong *in vitro* anti-staphylococci activity of this slug linked to bioproducts produced by an isolate identified as *Bacillus pumilus*. This evidence justified the interest on a deeper characterization of *B. pumilus* isolates, and their antimicrobial(s) singularity, due to the potential therapeutic or biotechnological interest, which were the main goals of this thesis. For this purpose, a collection of previously identified *B. pumilus* isolates from different origins was characterized at different levels (phylogeny, antimicrobial susceptibility, virulence and identification and characterization of peptides with potential antibacterial or anti-biofilm activity).

Phylogenetic analyses derived from *gyrB* and *rpoB* gene sequences determined the reclassification of most (93%, 38 of 41) isolates from the collection, which were now assigned to *B. safensis* or to a previously unrecognized clade. Moreover, distinctive phenotypic, genotypic and chemotaxonomic features of these isolates allowed the recognition of a new species within *B. pumilus* group, recovered from health's product contaminants in Portugal, and for which the name *Bacillus invictus* sp. nov. is proposed. In addition, it was demonstrated that these *B. pumilus* group species encompass a clonally diverse population, which can justify their high adaptability to different niches. Delineation of these *B. pumilus* group species was also supported by congruent protein profiles, assessed by matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and cellular composition by Fourier Transform Infrared Spectroscopy (FTIR), demonstrating the suitability of these methodologies for their rapid and low cost discrimination.

All *B. pumilus* group species revealed the absence of antibiotic resistance and virulence genes, which consist on safety features required for their application in the food and/ or pharmaceutical industries.

Most isolates of *B. pumilus* and *B. safensis* revealed surface-active properties compatible with biosurfactant compounds production. Analysis by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI/MS/MS) of these compounds revealed a mixture of antimicrobial peptides composed of specific pumilacidin and surfactin variants in *B. safensis*, whereas in *B. pumilus* only specific pumilacidin variants were detected.

In conclusion, most *B. pumilus* correspond to the less well known *B. safensis* and to a new species here proposed as *Bacillus invictus* sp. nov.. This taxonomic reassignment is congruent with the antimicrobial peptides types produced by these different species. Moreover, and beyond the contribution to accurately discriminate *B. pumilus* group species, the present work also demonstrates for the first time their genomic plasticity, which can justify their widespread to different environments and, consequently, its ability to produce this diverse array of distinct compounds with a broad range of activities.

Sumário

A natureza e a sua biodiversidade são uma fonte inesgotável de compostos naturais com estruturas químicas únicas, que possuem uma relevante atividade antimicrobiana. Compostos produzidos por microrganismos têm desempenhado um papel particularmente importante na descoberta de agentes com interesse terapêutico em doenças infeciosas.

O crescente aumento de infeções provocadas por bactérias resistentes a múltiplos antibióticos (MDR), com particular relevo para *Staphylococcus aureus* resistentes à meticilina (MRSA) e enterococos resistentes à vancomicina (VRE), tornou-se uma grande preocupação, pelo aumento de morbilidade e mortalidade que condicionam, assim como pelo impacto socioeconómico. Assim, é premente a necessidade de identificar e desenvolver novos compostos antibacterianos e estratégias alternativas de combate a infeções.

Durante várias décadas, em algumas regiões de Portugal, uma lesma foi utilizada como medicina tradicional para o tratamento de infeções cutâneas. Ensaios preliminares, *in vitro*, evidenciaram a presença neste gastrópode de um isolado de *Bacillus pumilus* com elevada atividade anti-estafilocócica. Este dado justificou o interesse numa caracterização mais detalhada de isolados desta espécie, assim como dos seus produtos, tendo em vista a potencial aplicação na prevenção e tratamento de infeções bacterianas. Assim, e decorrente da atividade antimicrobiana reportada, bem como da sua importância farmacêutica e biotecnológica, procedeu-se à caracterização de uma coleção de isolados de *B. pumilus* a diferentes níveis (filogenia, susceptibilidade a antimicrobianos, virulência e identificação e caracterização de péptidos com potencial atividade antibacteriana ou anti-biofilme).

A análise filogenética, tendo por base as sequências genéticas de *gyrB* e *rpoB*, determinou a reclassificação da maioria dos isolados (93%, 38 de 41) como *B. safensis* e a identificação de um novo grupo de isolados até então não reconhecidos. A caracterização fenotípica, genotípica e quimiotaxonómica permitiu o reconhecimento de uma nova espécie no grupo *B. pumilus*, num isolado encontrado em Portugal como contaminante de um medicamento, para o qual o nome *Bacillus invictus* sp. nov. foi proposto. Por outro lado, as espécies caracterizadas pertencentes ao grupo *B. pumilus* revelaram uma estrutura clonal diversificada, o que pode justificar a sua grande capacidade de adaptação a diferentes nichos.

A diferenciação destas espécies foi também suportada pelos perfis proteicos e de composição celular obtidos por MALDI-TOF/MS e FTIR, respetivamente, demonstrando a adequabilidade destas metodologias, rápidas e de baixo custo, na sua diferenciação.

Todos os isolados pertencentes ao grupo de *B. pumilus* revelaram ausência de genes de resistência a antibióticos e genes de virulência, características necessárias para assegurar a sua inocuidade aquando de uma possível aplicação na indústria alimentar e/ou farmacêutica.

A maioria dos isolados de *B. pumilus* e *B. safensis* estudados revelaram propriedades compatíveis com a produção de compostos biosurfactantes. A caracterização obtida por LC/ESI-MS/MS revelou a presença de uma mistura de péptidos antimicrobianos contendo variantes de pumilacidina e surfactina detetadas apenas em isolados de *B. safensis*, enquanto que em *B. pumilus* apenas uma variante especifica de pumilacidina foi verificada.

Decorrente deste estudo, a maioria dos isolados previamente identificados como *B. pumilus* são agora classificados como *B. safensis*, ou como pertencentes a uma possível nova espécie, a que foi proposta a designação de *Bacillus invictus* sp. nov. Verificou-se ainda que a reclassificação taxonómica é congruente com a os tipos de péptidos antibacterianos produzidos pelas diferentes espécies. Para além do contributo para a correta e rápida discriminação das espécies do grupo *B. pumilus*, o presente trabalho demonstra ainda, pela primeira vez, a plasticidade genómica destas bactérias, o que pode justificar a sua dispersão em diferentes ambientes e, consequentemente, a sua capacidade de produção de um conjunto diversificado de biocompostos, com uma ampla gama de atividades.

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List of Abbreviations

ACN	Acetonitrile
AMP	Antimicrobial peptide
AMPA	Antimicrobial Sequence Scanning System
ANN	Artificial Neural Network
APPA	Amino acid (Z)-l-2-Amino-5-Phosphono-3-Pentenoic Acid
ARDB	Antibiotic resistance genes database
aroE	Shikimate dehydrogenase gene
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLIS	Bacteriocin-like inhibitory substance
CAMP	Collection of Anti-Microbial Peptides database
CBD	Calgary biofilm device
CC	Complex Type
CCUG	Culture Collection of University of Göteborg
CD	Circular Dichroism
CFS	Cell-Free Supernatant
CFU	Colony Forming Unit
CHCA	α-cyano-4-hydroxycinnamic acid
CLSI	Clinical and Laboratory Standards Institute
CMC	Critical Micelle Concentration
CMV	Cytomegalovirus
CNS	Coagulase-negative *Staphylococci*
CytK	Cytotoxin K
Da	Dalton
DDH	DNA-DNA Hybridization
DHB	2,5-dihydroxybenzoic acid
DNA	Deoxyribonucleic Acid
DPG	DiphosphatIdylglycerol
DSM	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
E_{24}	Emulsification Index
EFSA	European Food Safety Authority
EPS	Extracellular Polymeric Substances
ESI	Electrospray Ionization

ESI-MS	Electrospray Ionization with Tandem Mass Spectrometry
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	Ferulic acid
FAME	Fatty Acid Methyl Ester
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FPLC	Fast Protein Liquid Chromatography
FTIR-ATR	Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance
GCg	Genomic G+C content
GISA	Glycopeptide-intermediate-susceptible *Staphylococcus aureus*
gyrB	β-subunit of DNA gyrase gene
Hbl	Hemolysin BL
HCA	Hierarchical Clustering Analysis
HCl	Chloridric Acid
HIV	Human immunodeficiency virus
IL	Interleukin
JTT	Jones-Taylor-Thornton
Kb	Kilobase
kDa	KiloDalton
LAB	Lactic acid bacteria
LB	Luria Bertani-Miller medium
LC/ESI-MS/MS	Liquid Chromatography – Electrospray Tandem Mass Spectrometry
LinR	Linezolid Resistant
LinS	Linezolid-Susceptible
LPBS	Lipopeptides biosurfactants
LZD-R	Linezolid-Resistant *Staphylococcus epidermidis*
MALDI-TOF/MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
MBEC	Minimal Biofilm Eradication Concentration
MDR	Multidrug Resistant
MEL	Mannosylerythritol lipid
MH	Mueller Hinton agar
MIC	Minimal Inhibitory Concentration
MIS	Sherlock Microbial Identification System
MK	Menaquinone
ML	Maximum-Likelihood

MLST	Multilocus Sequence Typing
mN/m	millinewton/meter
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MRSE	Methicillin-Resistant *Staphylococcus epidermidis*
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
mutL	DNA mismatch repair protein gene
MW	Molecular Weight
NaCl	Sodium chloride
NASA	National Aeronautics and Space Administration
Nhe	Non-hemolytic enterotoxin
NJ	Neighbour-Joining
NRP	Nonribosomal peptide
NRPS	Non-ribosomal peptide synthetase
NTD	3,3´-neotrehalosadiamine
OD	Optical Density
ORF	Open reading frame
PAS	Periodic Acid-Schiff
PBP	Penicilin-Binding-Proteins
PBS	Phosphate buffer saline
PC	Principal Component
PCA	Principal Component Analysis
PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
PFGE-types	Pulsed-Field Gel Electrophoresis-types
PG	Phosphatidylglycerol
PGPR	Plant Growth-Promoters
PK	Polyketides
PKS	Polyketide synthetase
PLSDA	Partial Least Square Discriminant Analysis
PMF	Peptide Mass Fingerprinting
PRSP	Penicillin-resistant *Streptococcus pneumoniae*
PVDF	Polyvinylidene Fluoride Membrane
pycA	Pyruvate carboxylase A gene
pyre	Orotate phosphoribosyltransferase gene
QPS	Qualified Presumption of Safety

RF	Random Forests
RNA	Ribonucleic acid
RP-HPLC	Reverse-phase high-performance liquid chromatography
rpoB	β-subunit of RNA polymerase gene
Rt	Retention Time
rRNA	Ribosomal RNA
SA	Sinapinic acid
SAR	Systemic Acquired Resistance
SASP	Small Acid-Soluble Spore Protein
SCAN	Scientific Committee on Animal Nutrition
SCCmec	Staphylococcal Cassette Chromosome *mec*
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SNAC	S-N-acetyl cysteamine
ST	Surface Tension
Ste1	Methicillin- and Linezolid-Resistant *Staphylococcus epidermidis*
SVM	Support Vector Machine
TEII	Type II thioesterases
TFA	Trifluoroacetic Acid
trpB	Tryptophan synthase gene
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
UPGMA	Unweighted-Pair Group Method using Average linkages
US FDA	United States Food and Drug Administration
VRE	Vancomycin-Resistant Enterococci
γ-PGA	Poly-γ-Glutamic Acid

Research Aims

The intensive use and misuse of antibiotics have generated a strong selective pressure for the emergence of multi-drug resistant bacteria, which stimulates efforts to identify and develop new antibacterial compounds from natural sources or alternative anti-infectious strategies

During several decades, in some regions of Portugal, a terrestrial black slug (*Arion ater*) was used as a traditional medicine in the treatment of cutaneous infections, including wounds and abscesses. Previous work demonstrated a strong *in vitro* anti-staphylococci activity of this slug throughout the contribution of bioproducts produced by an isolate identified as *Bacillus pumilus*. Despite previous reports on the production of a molecule with a putative anti-staphylococci activity by *B. pumilus*, no known application has been recognized. Moreover, the recently disclosed diversity within this species suggests a neglected potential of bioproducts produced by species from the *B. pumilus* group. In the present work, and due to the difficulty to accurately distinguish *B. pumilus* and its closely related counterparts, we deeply characterized an extended collection of isolates previously identified as *B. pumilus*.

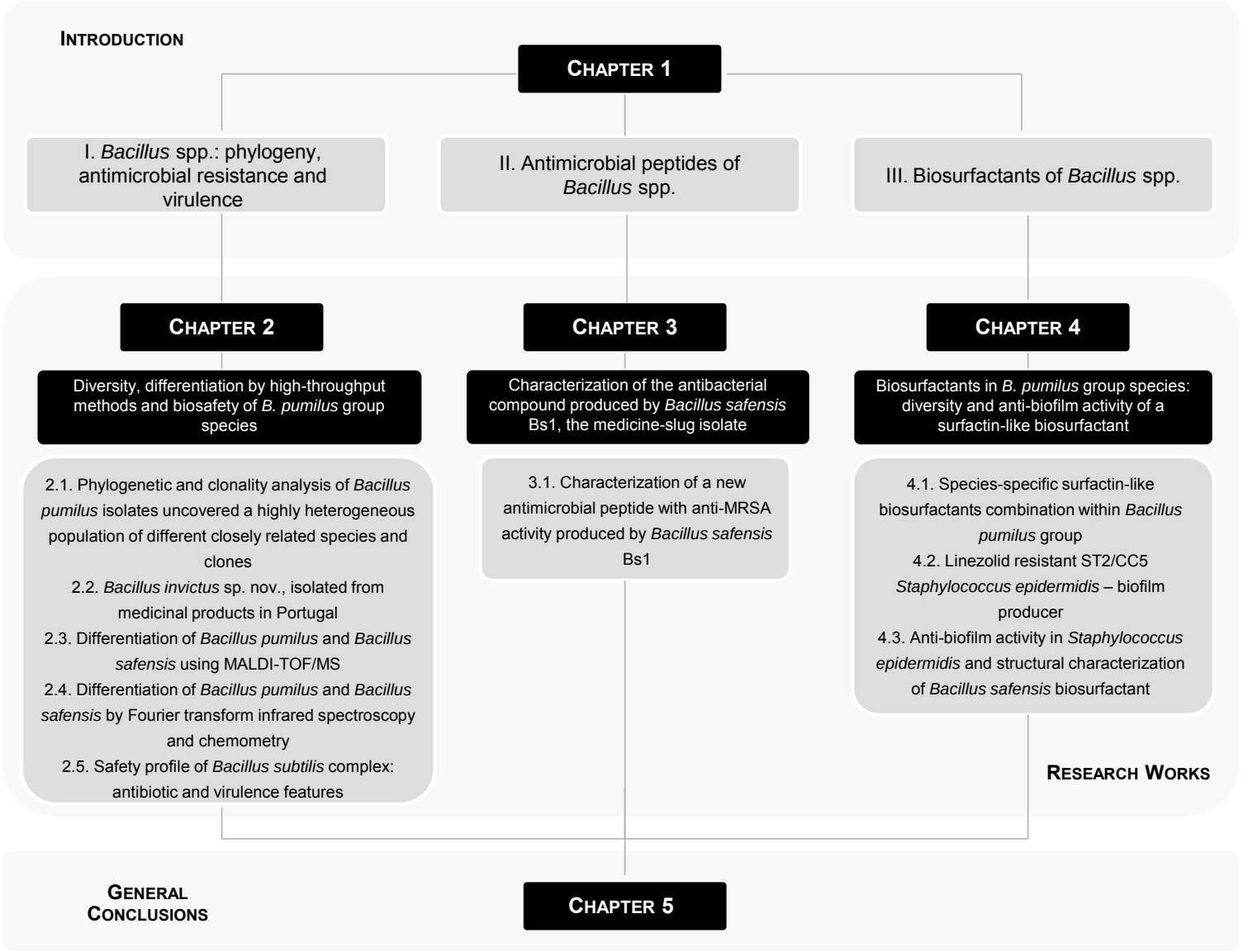
Our **hypothesis** consists on that *the medicine-slug isolate corresponds to an Arion ater adapted clonal lineage that produces antimicrobial compound(s) with anti-staphylococci activity, which might be unusual among B. pumilus group strains. These antimicrobial properties confer to the medicine-slug isolate or to its anti-stapphylococci compound(s) a pharmaceutical or biotechnological interest.*

The **global objective** of this proposal is to assess the singularity of the medicine-slug isolate and to characterize its antimicrobial compound(s).

The **specific objectives** are:

- To position phylogenetically the medicine-slug isolate, evaluating its niche-clonal specificity.
- To investigate the potential of high-throughput approaches such as Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF) and Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR), coupled with chemometric tools, to discriminate within *B. pumilus* group species.
- To investigate, in the slug-medicine isolate and closely related species, the presence of safety features (antibiotic resistance and virulence) required for their application in humans and agriculture.
- To characterize the antibacterial compound(s) produced by the medicine-slug isolate.
- To evaluate the diversity and anti-adhesive or anti-biofilm properties of biosurfactant compounds produced in slug-medicine isolate and its closely related species, and to provide biochemical characterization of these surface-active compounds.

Outline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Outline

The structure of this thesis is organized in a sequential order of work and the rational integration of each chapter is described as follows:

Chapter 1 presents an overview of the state of the art of the contents involved in the study, which is divided in three sections. In section I the diversity and phylogenetic positioning of *B. pumilus* group members, as well as fundaments of high-throughput methodologies for bacterial identification were addressed. Moreover, *B. pumilus* group members' pathogenicity, virulence, antibiotic resistance and biotechnological potential were also reviewed. Section II addresses the the diversity and spectrum of activity of different class of antimicrobial peptides produced by *Bacillus spp.* Finally, section III, properties, diversity, distribution and the potential applications of biosurfactants produced by *Bacillus* spp. are reviewed.

Chapter 2, 3 and 4 presents the findings that would answer the specific aims of the thesis. Such results have been organized throughout the papers (n=9; 1 publication in peer review scientific journal, 4 manuscripts submitted, 2 in preparation for publication and and 2 manuscripts awaiting patentability assessment).

In Chapter 2, the **diversity, differentiation by high-throughput methods and biosafety of *B. pumilus* group species** is presented throughout the following papers:

- R. Branquinho, L. Meirinhos-Soares, J. A. Carriço, M. Pintado, L. V. Peixe. 2014. Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a highly heterogeneous population of different closely related species and clones. FEMS Microbiology Ecology *(submitted).*
- R. Branquinho, C. Sousa, H. Osório, L. Meirinhos-Soares, J. Lopes, A. Abdulmawjood, G. Klein, H-J. Busse, P. Kämpfer, M. E. Pintado, L. V. Peixe. 2014. *Bacillus invictus* sp. nov., isolated from healthy products in Portugal (manuscript in preparation to International Journal of Systematic and Evolutionary Microbiology).
- R. Branquinho, C. Sousa, J. Lopes, M. E. Pintado, L. V. Peixe, H. Osório. 2014. Differentiation of *Bacillus pumilus* and *Bacillus safensis* using MALDI-TOF/MS. Journal of the American Society for Mass Spectrometry *(submitted).*

- C. Sousa, R. Branquinho, J. Lopes, L. Peixe. 2014. Differentiation of *Bacillus pumilus* and *Bacillus safensis* by Fourier transform infrared spectroscopy and chemometry. Journal of Food Microbiology *(submitted).*
- R. Branquinho, J. Pires, M. Amorim, M. E. Pintado. 2014. Safety profile of *Bacillus subtilis* complex: antibiotic and virulence features (manuscript in preparation).

Chapter 3, **Characterization of the antibacterial compound produced by *Bacillus safensis* Bs1, the medicine-slug isolate** is presented**.** Results were organized in the paper:

- R. Branquinho, H. Osório, P. Pereira, M. E. Pintado, L. V. Peixe. 2014. Characterization of a new antimicrobial peptide with anti-MRSA activity produced by *Bacillus safensis* Bs1 (manuscript in preparation - *Patentability under evaluation*).

In Chapter 4, **Biosurfactants in *B. pumilus* group species: diversity and anti-biofilm activity of a surfactin-like biosurfactant,** addresses the screening, diversity and chemical identification of tension-active compounds produced by *B. pumilus* group members, also including the assessment of a *B. safensis* biosurfactant as an agent preventing biofilm formation of *S. epidermidis* is also presented. Results were organized in the following papers:

- R. Branquinho, L. V. Peixe, J. Pires, M. E. Pintado, M. G. Martinotti, L. Fracchia, G. Allegrone. 2014. Species-specific surfactin-like combinations of biosurfactants within *Bacillus pumilus* group. Bioresource Technology *(submitted).*
- M. Barros, R. Branquinho, F. Grosso, L. Peixe, C. Novais. 2014. Emergence of Methicillin and Linezolid Resistant ST2/CC5 *Staphylococcus epidermidis,* Portugal 2012: First Cases Report. Emerging Infectious Diseases (*In Press*).
- R. Branquinho, J. Pires, G. Allegrone, M. E. Pintado, L. Fracchia, L. V. Peixe. 2014. Anti-biofilm activity in *Staphylococcus epidermidis* and structural characterization of *Bacillus safensis* biosurfactant (manuscript in preparation - *Patentability under evaluation*).

Highlighted results from these studies are reflected in the general conclusions described in Chapter 5.

Additionally, the results presented in this thesis have also been partially presented in national and international scientific meetings and conference proceedings, as follows:

Chapter 2:

- R. Branquinho, C. Sousa, H. Osório, L. Meirinhos-Soares, J. Lopes, A. Abdulmawjood, G. Klein, M. E. Pintado e L. Peixe. 2013. *Bacillus invictus* sp. nov., a new species isolated from medicinal products in Portugal. Poster presentation at MicroBiotec 2013 (Book of abstracts pp.151), Aveiro, Portugal.
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Chapter 4:

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- R. Branquinho, M. M. E. Pintado e L. Peixe. 2010. Diversity of non-ribosomal lipopeptides in *Bacillus pumilus* from different sources. Poster presentation at ICAR 2010 (Book of abstracts pp. 537), Valladolid, Spain.

CHAPTER 1
INTRODUCTION

This chapter contains the state of the art on the phylogeny, virulence and antibiotic resistance aspects of interest to *Bacillus pumilus* group, the subject of this thesis. Its general properties and relevance are reviewed, with special emphasis on their diversity, peptides with antimicrobial activity and biosurfactants. Furthermore, important human and environmental safety features will be discussed.

I. *Bacillus spp.*: phylogeny, antimicrobial resistance and virulence

1. *Bacillus* spp.

Members of the genus *Bacillus* (Kingdom Bacteria; Phylum Firmicutes; Class Bacilli; Order Bacillales; Family Bacillaceae) include Gram-positive, spore-forming, rod-shaped, aerobic or facultative anaerobic bacteria, which are phenotypically and genotypically heterogeneous (Earl et al., 2008; Satomi et al., 2006). This polyphyletic genus was discovered by Cohn in 1872 (Cohn, 1872), includes currently near two hundred species (Parte, 2013), and has been subjected to frequent restructuration's with the continuous recognition of new species (Lai et al., 2014; Liu et al., 2013; You et al., 2013; Shivaji et al., 2006).

Phylogenetically, Ash et al. (1991) clustered *Bacillus* species into five RNA groups (RNA group 1, RNA group 2, RNA group 3, RNA group 4 and RNA group 5), and more recently, Nielsen et al (1995) reported the presence of an additional RNA group (RNA group 6) for alkaliphilic and alkalitolerant species (Nielsen et al., 1995). For the purpose of this thesis, only RNA group 1, where *Bacillus pumilus* group species are included, will be detailed. The phylogenetic affiliation of *Bacillus* spp. and other related

Gram positive representative species, based on 16S rRNA gene is shown in Figure 1 (Felsenstein, 1993).

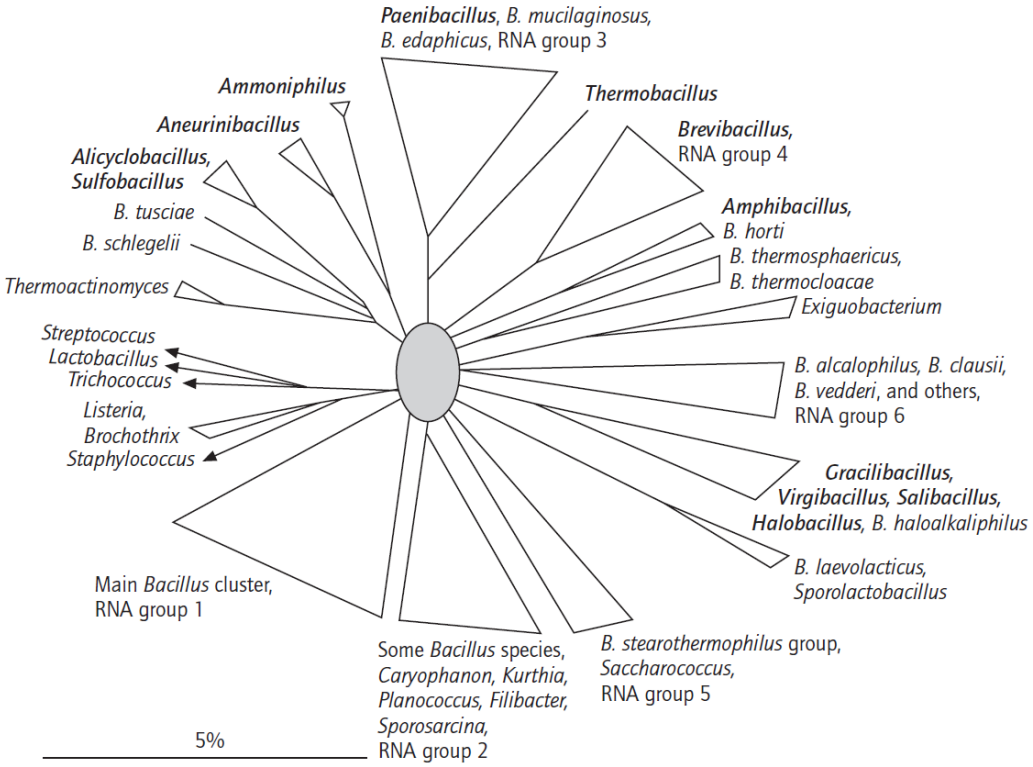


Figure 1. Schematic outline of the phylogenetic relationships between aerobic representatives of Gram-positive bacteria based on 16S rRNA gene sequences. Areas of the triangles represent closeness's of the number of species included in the taxa covered by the triangle. The circle indicates the uncertainty of the order at which the lineages diverge from each other. Adapted from (Berkeley et al., 2008).

A more detailed phylogenetic analysis within *Bacillus* members, based upon the neighbor-joining method is illustrated in Figures 2 and 3.

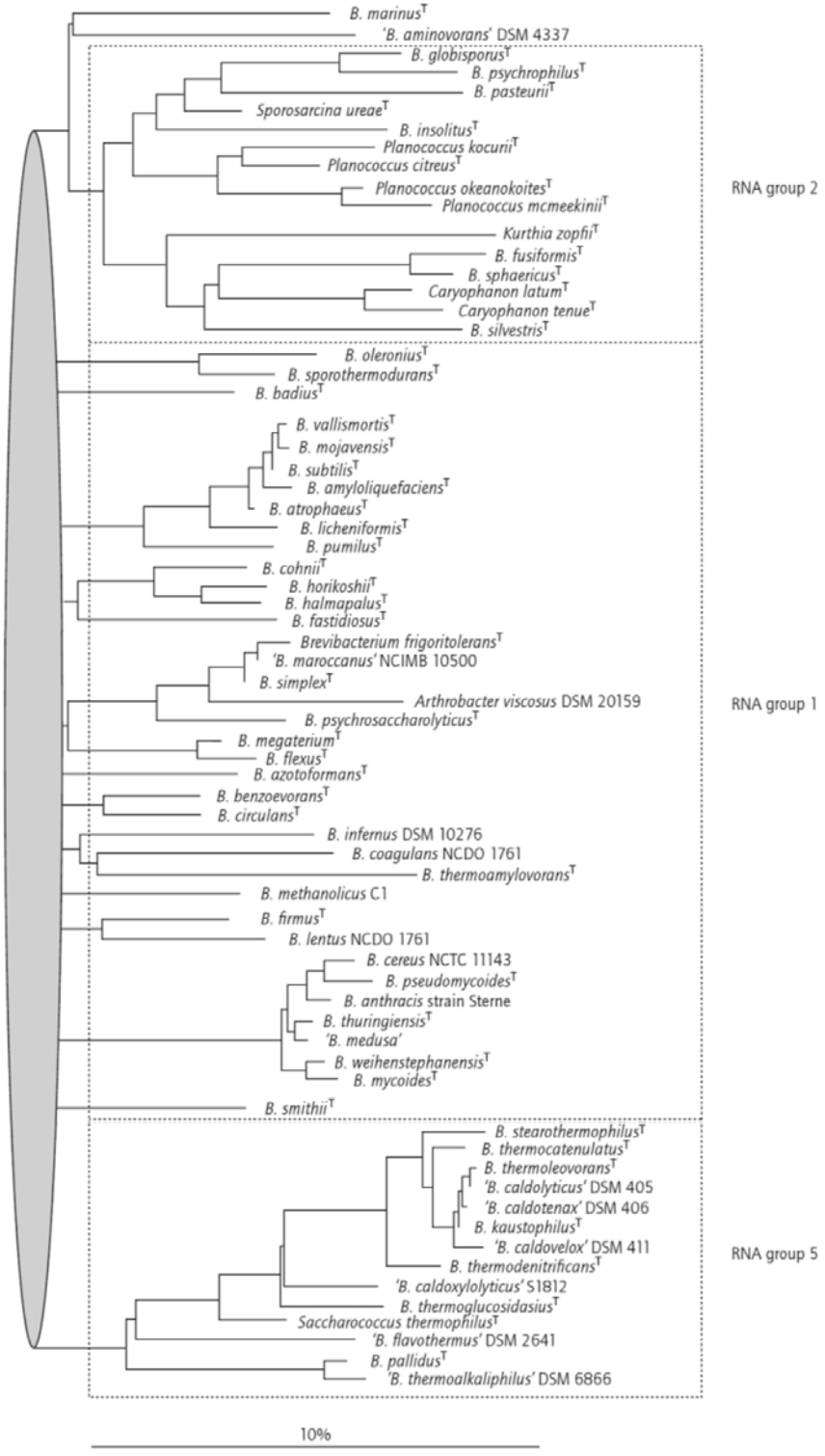


Figure 2. Detailed neighbour-joining tree of *Bacillus* species of RNA groups 1, 2 and 5. The dotted area indicates the uncertainty of the order at which the lineages diverge from each other. The area was chosen somewhat arbitrarily and cover more recent branching points. The bar indicates 10% nucleotide substitutions. B, *Bacillus*; T, type strain. (Adapted from: Berkeley et al., 2008).

Figure 3. Detailed neighbor-joining tree of *Bacillus* species members of RNA groups 3, 4 and 6, other *Bacillus* species, and lineages containing reclassified *Bacillus* species. The dotted areas indicate the uncertainty of the order at which the lineages diverge from each other. The areas are chosen somewhat arbitrarily and may more recent branching points. The bar indicates 10% nucleotide substitutions. Gra, *Gracilibacillus*; Amb, *Amphibacillus*; B, *Bacillus*; Bv, *Brevibacillus*; Ex, *Exiguobacterium*; Hb, *Halobacillus*; Pb, *Paenibacillus*; Sb, *Salibacillus*; T, Type strain; Vg, *Virgibacillus*. (Adapted from: Berkeley et al., 2008).

1.1. *Bacillus* RNA group 1

This group constitutes the core of *Bacillus* spp. and displays the name of one of the first organisms of which the complete genome was published (Kunst et al., 1997) - *Bacillus subtilis* (Figure 2). This group is highly heterogeneous, encompassing several consistent clusters and single-species lineages, including those of the *Bacillus pumilus* group. One of the subgroups that embraces *Bacillus* RNA group I encloses *B. subtilis* relatives, which actually encompasses 19 species including, *Bacillus subtilis* subs. *subtilis, B. vallismortis, B. mojavensis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*, *B. pumilus,* presented in Figure 2, whereas the species most recently discovered *B. subtilis* subsp. *inaquosorum*, *B. subtilis* subs. *spizizenni, B. tequilensis*, *B. sonorensis*, *B.axarquiensis*, *B. malacitensis*, *B. velezensis*, *B. safensis, B. aerophilus*, *B. stratosphericus*, *B. altitudinis* and *B. xiamenensis* are not (Jeyaram et al., 2011; Earl et al., 2008)

A second subgroup encompasses *B. cohnii*, *B. horikoshii* and *B. halmapalus*. A third one harbors *B. cereus*, *B. pseudomycoides*, *B. anthracis*, *B. thuringiensis, B. weihenstephanensis*, *B. mycoides* and a novel thermotolerant species, *B. cytotoxicus* (Guinebretière et al., 2013), which can be compiled under the term *B. cereus sensu lato* or *B. cereus group* (Ehling-Schulz & Messelhaeusser, 2013; Kolsto et al., 2009). A fourth subgroup embraces *B. simplex* and *B. psychrosaccharolyticus*. Based on the reported characteristics, RNA group 1 can also group other bacterial genera as *Virgibacillus*, *Salibacillus*, *Halobacillus* and *Gracilibacillus* (Figure 3).

Phenotypically, all these *Bacillus* species produce acids from a wide range of sugars and also present oval endospores, which are generally located centrally/subterminally (Leuschner, 2003). Moreover, some species such as *B. cereus* and *B. liqueniformis* are facultative anaerobes.

2. *Bacillus pumilus* group

2.1. General features

Bacillus pumilus group, encompasses *B. pumilus*, the first species described, and the closely related species *B. safensis*, *B. aerophilus*, *B. stratosphericus*, *B. altitudinis* and *B. xiamenensis* (Lai et al., 2014; Liu et al., 2013; Satomi et al., 2006; Shivaji et al., 2006). These bacteria are readily isolated from diverse natural envirnments such as soil and

marine settings, plants and air of high altitudes (Lai et al., 2014; Liu et al., 2013; Freitas et al., 2008; Satomi et al., 2006; Shivaji et al., 2006). General morphologic characteristics of these species include Gram-positive, rod-shaped, aerobic growth and the presence of spore located terminally or sub-terminally (Berkeley et al., 2008).

This group has a high economic relevance, derived from the wide range of applications of these microorganisms or their products into biotechnological, environmental and biopharmaceutical purposes (Pérez-García et al., 2011; Aunpad & Na-Bangchang, 2007; Hong et al., 2005; Sanders et al., 2003; Lehman et al., 2001), which will be further detailed in sub-section 4. Although rarely, *B. pumilus* group species have also been associated with food poisonings and human infections, mainly in immunocompromised patients, including anthrax-like cutaneous lesions (Johnson et al., 2008; Bentur et al., 2007; From et al., 2007a; Tena et al., 2007; Haymore et al., 2006; Ozkocaman et al., 2006; Castagnola et al., 2001) (see sub-section 3, for more detailed information)

The *B. pumilus* species, firstly designated in 1901 (Gottheil, 1901) has been the most extensively described member of this group (Boone et al., 2005), being described from soils, plants, foods, health products, cosmetics, clean room environments, water and even animals (Liu et al., 2013; Branquinho et al., 2012; Ouoba et al., 2004; Wei et al., 1996). Nevertheless, only one genome is completely sequenced (*B. pumilus* SAFR-032 strain, isolated from the Spacecraft Assembly Facility at National Aeronautics and Space Administration (NASA)), comprising 3.7 Mb and coding for 3679 proteins.

A large range of applications has been attributed to *B. pumilus*, mainly related with the production of peptides with antimicrobial activity or its use as animal and human probiotics (EFSA, 2011; Hong et al., 2005; Sanders et al., 2003), phytosanitary products (Pérez-García et al., 2011) or plant growth promoters (Joo et al., 2005). In addition, although infrequently, it has also been reported their involvement in human and animal diseases (Kimouli M et al., 2012; Johnson et al., 2008; Bentur et al., 2007; From et al., 2007a; Callegan et al., 2006; Haymore et al., 2006; Ozkocaman et al., 2006; Galanos et al., 2003; Castagnola et al., 2001; Peltola et al., 2001; Turnbull, 1997).

B. safensis was initially reported by NASA as one of the major contaminants in spacecraft, associated with contamination of clean room assembly-facility surfaces due to its highly resistance to gamma and UV radiation (Satomi et al., 2006). More

recently it was identified in African oil and locust bean seeds for the production of fermented food condiments (Agbobatinkpo et al., 2013; Ahaotu et al., 2013), plant rhizosphere and from marine environments (Liu et al., 2013). Nevertheless, only one partially sequenced genome (3.68 Mb) of *B. safensis* VK strain, isolated from the rhizosphere of a cumin plant growing in the saline desert of India is available (Kothari et al., 2013).

B. altitudinis, primarily isolated from cryogenic tubes used for collecting air samples from high altitudes (Shivaji et al., 2006), seems to be the most common species from this group in marine environments (Liu et al 2013). No information of complete genome sequences is available.

B. stratosphericus and *B. aerophilus* were originally isolated from air samples (Shivaji *et al.,* 2006). Soils and estuarine sediments were also reported as habitats for *B. stratosphericus* (Zhang et al., 2012; Yadav et al., 2011). Nevertheless, the type strai of these species are no longer available in public collections.

Finally, *B. xiamensis* was recently identified (Lai et al., 2014) in the intestinal tract contents of a flathead mullet, *Mugil cephalus*, captured from the sea off Xiamen Island, China. One draft genome of strain HYC-10, containing 3.6 Mb and comprising 3687 protein-coding genes is available.

2.2. Evolution and phylogenetic boundaries

Many methods have been applied to identify and discriminate among closed related species, as *B. pumilus* group members. Beyond the classical morphological, physiological and biochemical characteristics, phylogenetic analysis based on single or multilocus sequence typing (MLST) of housekeeping genes, have been frequently used to depict phylogenetic affiliations among members of *Bacillus* genus (Helgason et al., 2000).

2.2.1 Phenotypic based phylogeny

Members of the *B. pumilus* group present very similar phenotypic characteristics, which hinders its identification merely based on biochemical and/or morphological properties. Nevertheless, some distinct aspects related with carbohydrates and amino acid metabolization profiles can be assigned, as summarized in Table 1.

Table 1. Biochemical and physiological characteristics of *B. pumilus* group species.

	***B. pumilus* ATCC 7061^T**	***B. safensis* FO-036b^T**	***B. xiamenensis* HYC-10^T**	***B. altitudinis* 41KF2b^T**
Temperature range (optimum) (ºC)	30	30	30–37	30–37
NaCl range (optimum) (%, w/v)	1–3	1–3	1–3	1–3
pH range (optimum)	6–8	6–8	6–8	6–8
Carbohydrates acid production profile				
Cellobiose	-	+	+	+
D-Arabinose	+	+	-	+
Erythritol	+	+	-	-
Glucose	-	+	-	-
Inositol	+	-	-	-
Maltose	+	-	-	+
Mannitol	+	+	+	+
Mannose	+	-	+	+
Raffinose	+	-	+	-
Rhamnose	-	-	+	+
Sorbitol	-	-	+	+
Starch	-	-	+	+
Amino acid utilization				
L-Arginine	-	-	-	+

2.2.2 Genotypic based phylogeny

B. pumilus group species present a remarkable high level of 16S rRNA gene similarity (>99%) (Liu et al., 2013; Satomi et al., 2006). In fact, limitations of 16S rDNA sequences to decipher relationships at species level have been recognized as a result of its conserved nature for different species (Adékambi et al., 2008; Wang et al., 2007; Konstantinidis & Tiedje, 2005;). In fact, strains belonging to *B. subtilis* group showing ≥99% 16S rDNA sequences similarity, may not belong to the same species. A phylogenetic tree based on the 16S rRNA genes of marine bacteria belonging to the *B. pumilus* group is presented in Figure 4.

Despite the high similarity observed for 16S rDNA sequences between the isolates analized, *B. pumilus* members from marine settings are divided into two groups: i) the *B. altitudinis* and *B. xiamenensis* and ii) *B. pumilus* and *B. safensis* (Liu et al., 2013).

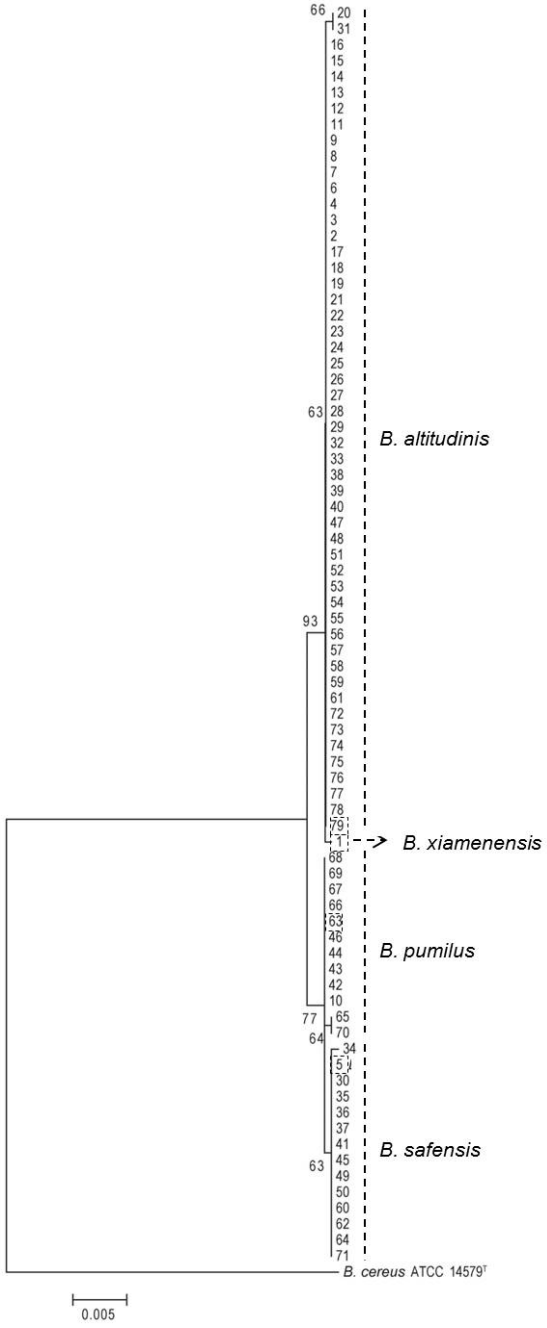


Figure 4. Phylogenetic affiliation based on 16S rRNA genes of *B. pumilus* group members recovered from marine sources. The tree was constructed using the neighbor-joining method. Bootstrap values over 50% (1000 replications) were shown at each node. Bar, % estimated substitution. *B. cereus* ATCC 14579^T was used as outgroup. (Adapted from: Liu et al., 2013).

The ability to order prokaryotic taxa hierarchically has been constantly improved by *ad hoc committee* (Stackebrandt et al., 2002). In fact, current consensus determines that an informative level of phylogenetic data would be obtained from the determination of a minimum of five protein-coding gene sequences, which should be diverse at chromosomal loci and widely distributed among taxa and, for which, extensive DNA±DNA intraspecific diversity has been evaluated (Stackebrandt et al., 2002). It consists on an extension of the MLST approach, increasingly used for the indexing and organizing of within-species genetic variability, which has not been extensively applied to *Bacillus* species other than *B. cereus* group members.

In which concerns *B. pumilus* group species, recently Liu et al. (2013) proposed the use of 7 concatenated housekeeping genes, namely *gyrB* (β-subunit of DNA gyrase), *rpoB* (β-subunit of RNA polymerase), *pycA* (pyruvate carboxylase A), *pyre* (orotate phosphoribosyltransferase), *mutL* (DNA mismatch repair protein), *aroE* (shikimate dehydrogenase) and *trpB* (tryptophan synthase) (Figure 5) to depict taxonomic affiliations among *B. pumilus*, *B. safensis*, *B. altitudinis* and *B. xiamenensis* isolates recovered from marine environments. Their results evidenced that phylogenetic trees obtained from individual genes and concatenated genes exhibited similar topology structures, suggesting that, with the exception of *rpoB*, these genes are appropriate to depict phylogenetic affiliations within species of this group and that the taxonomic status of these species is far to be completely known.

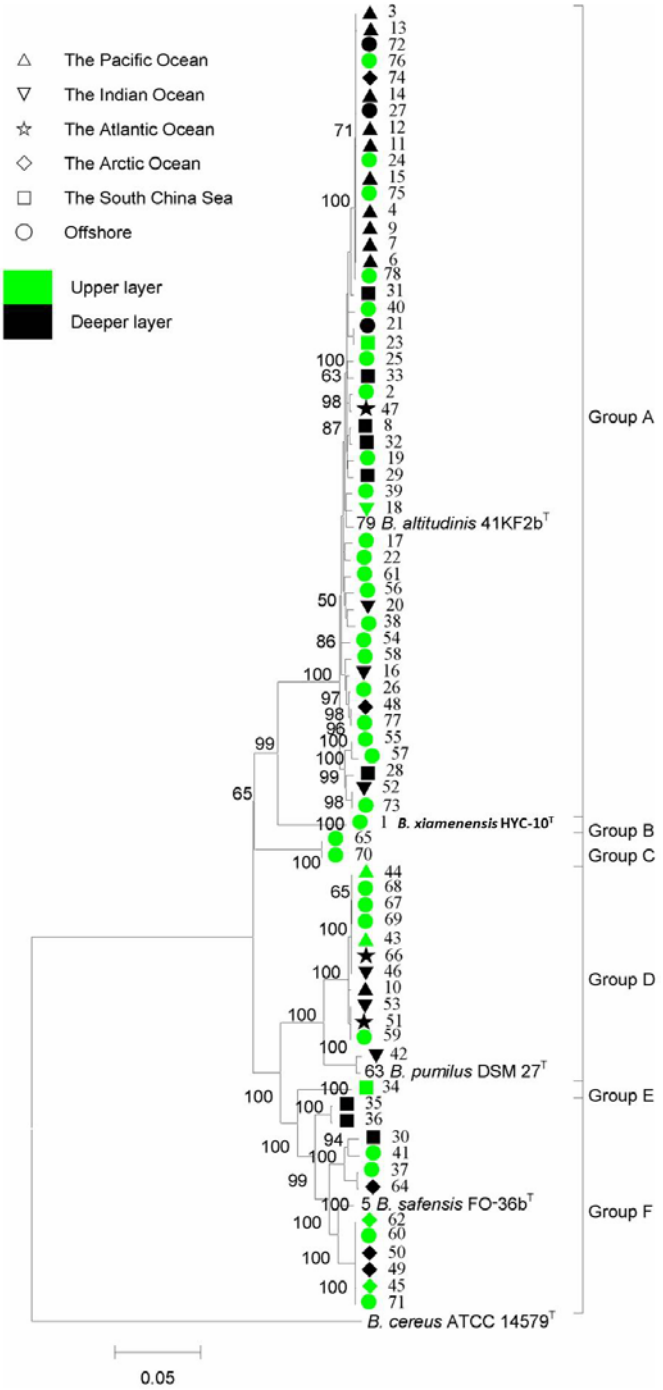


Figure 5. Phylogenetic tree based on seven concatenated housekeeping genes of marine *B. pumilus* group isolates. Groups represent: (A) *B. altitudinis*, (B) *B. xiamenensis*, (D) *B. pumilus*, (F) *B. safensis* and (C and E) could not be assigned. Tree constructed using the neighbor-joining method. Bootstrap values over 50% (1000 replications) were shown at each node. Bar, % estimated substitution. *B. cereus* ATCC 14579^T was used as the outgroup. (Adapted from: Liu et al., 2013).

Still, using this criterion, important aspects regarding the conditions in which different groups of bacteria diverge from each other, and also which molecular, biochemical, structural or physiological properties of *Bacillus pumilus* group species allow their discrimination, remain to be understood. Moreover, cut-off values for its randomization still remain unclarified.

In agreement with Gevers et al., (2006) any effort to produce a robust definition of species is hindered by the effect of biological processes and divergence between species (Gevers et al., 2006). Therefore, it is important to develop other means to clarify these critical aspects of microbial evolution (Gupta & Griffiths, 2002). Indeed, the markers that are ideally suited for evolutionary/taxonomic studies should be homologous apomorphic characters that evolved only once (synapomorphy) during the course of evolution (Stackebrandt, 2006). The presence or absence of these markers in orthologous sequences should also be readily discernible and it should be minimally affected by factors as long-branch attraction effect, differences in evolutionary rates and horizontal gene transfer (Delsuc et al., 2005).

More recently, whole-genome sequencing analysis delivered new metrics to evaluate the taxonomic relationships among bacterial species (Chan et al., 2012). In this sense, ANI (average nucleotide identity) values ≥ 95% correspond to the traditional 7% DDH threshold (Goris et al., 2007; Konstantinidis & Tiedje, 2005). However, the scarce availability of whole genome sequences of *B. pumilus* members hinders their identification and differentiation.

2.2.3. Chemotaxonomic based phylogeny

Chemotaxonomic analysis enables differentiation of bacteria by studying their cell components, as fatty acids, membrane and cellular proteins, polysaccharides and/or nucleic acids (Herbel et al., 2013), characteristic components of each particular species, allowing their differentiation. Among chemotaxonomic methods used for strain differentiation, different spectroscopic-based methods such Matrix-assisted Laser Desorption/Ionization with Time-of-Flight mass spectrometry (MALDI-TOF/MS) (Conway et al., 2001; Claydon et al., 1996) and Fourier-Transformed Infrared Spectroscopy (FTIR) (Oberreuter et al., 2002; Helm et al., 1991a; Helm et al., 1991b) are increasingly being used for bacterial differentiation at species and subspecies levels (Novais et al., 2014; Sousa et al., 2014; Sousa et al., 2013; Vaz et al., 2013; Sousa et al., 2012; Wolters et al., 2011; Barbuddhe et al., 2008).

These methods are also recommended by *ad hoc committee* (Stackebrandt et al., 2002) but they have scarcely been applied to *B. pumilus* group members.

2.2.3.1 Application of MALDI-TOF MS in *Bacillus* systematics

MALDI-TOF MS is a fingerprinting approach that relies on the reproducible detection of large molecules which can be used for microbial identification, at different taxonomical levels (Clark et al., 2013; Keys et al., 2004), by comparing experimental mass spectra with a library of known reference strains or by comparing information of species-specific biomarkers identities (Clark et al., 2013; Lartigue, 2013; Emonet et al., 2010; Mellmann et al., 2009). It has emerged as an accurate, fast, robust, sensitive and low cost technique for characterization of large molecules, which has significantly impacted bacterial identification procedures, especially in clinical microbiology laboratories (Clark et al., 2013).

This mass spectroscopic technique is based on desorption and ionization of large molecules from a target plate with the aid of a matrix solution. The ionized molecules fly through the MALDI tube and reach the detector according to their size being the obtained spectra a graphical representation of the intensity versus the mass-to-charge ratio of the detected molecules. Concerning microbial typing, MALDI-TOF MS is mainly used for protein fingerprinting and the obtained mass spectra reflect the peptide and/or protein bacterial composition, most of which are ribosomal (Clark et al., 2013; Fenselau & Demirev, 2001). The quality of the mass spectra obtained for chemotaxonomic analysis depends on different factors such as culture media components, batch and manufacturer, bacterial growth time or matrix used (Chen et al., 2008) and requires strict standardization and optimization protocols. The most common matrices used are α-cyano-4-hydroxycinnamic acid (CHCA) (Williams et al., 2003), ferulic acid (FA) (Dickinson et al., 2004), sinapinic acid (SA) (Donohue et al., 2006; Lee et al., 2003) or 2,5-dihydroxybenzoic acid (DHB) (Moura et al., 2008), which favour the visualization of different types of biomolecules.

The obtained mass spectra can be directly compared with previously established databases to bacterial species identification (Clark et al., 2013; Sandrin et al., 2013) or used to identify species specific biomarkers through protein databases (e.g. MASCOT (Perkins et al., 1999) and SEQUEST (Eng et al., 1994)). Additionally, a multi variate data analysis approach, usually designated by chemometry, can be used

allowing the establishment of mathematical models through which more detailed information about the microorganisms can be extracted. This approach makes use of some chemometric models (Sousa et al., 2012; Naes, 2002; Alsberg et al., 1998) such as principal component analysis (PCA), hierarchical clustering analysis (HCA) and partial least square discriminant analysis (PLSDA) to explain spectral differences among bacterial species and/or clones.

MALDI-TOF MS has already been used in *Bacillus* spp. for the identification of *B. anthracis* and its close relatives (Hotta et al., 2011; Lasch et al., 2009), of *B. subtilis*, *B. cereus* and *B. licheniformis* (Fernandez-No et al., 2013). Nevertheless, scarce information is available considering isolates from *B. pumilus* group. Preliminary studies evaluating the discriminatory ability of MALDI-TOF MS in these species included only a few isolates from some species and/or were not conclusive (Farfour et al., 2012; Bohme et al., 2011; Dickinson et al., 2004;).

Concerning the identification of species-specific biomarkers in *Bacillus* spp., some reports showed that beyond ribosomal proteins, small acid-soluble spore proteins (SASPs) from spores could potentially be used as species-specific biomarkers. These proteins are generally highly conserved within and between the various species of *Bacillus* (Dybwad et al., 2013).

The rapid and accurate identification of proteins/peptides from mass spectra can only be achieved by using TOF/TOF devices (providing higher resolution of mass/charge), bioinformatic tools and databases.

Nevertheless, further and detailed studies are required to identify and elucidate these protein biomarkers in *B. pumilus* group members and reduce the difficulties of spectral interpretation. The increasing availability of bacterial genome information should afford a basis for the attribution of chemical structures to some of the biomarkers signals detected.

2.2.3.2 Application of FTIR in *Bacillus* Systematics

FTIR is a vibrational spectroscopic technique, based on the interaction of infrared radiation with a sample, providing a specific fingerprint that reflects the structure and composition of the whole cell (Blum & John, 2012; Naumann et al., 1991). It represents a non-destructive, fast, easy to use, and highly sensitive method for bacterial

characterization and identification, also providing information about bacterial metabolism (Becker et al., 2006), growth phase (Al-Qadiri et al., 2008) and antibiotic resistance determinants (Amiali et al., 2008). The infrared spectra reflects bacterial composition of lipids (3000–2800 cm^{-1}), proteins/amides I and II (1700–1500 cm^{-1}), phospholipids/DNA/RNA (1500–1185 cm^{-1}), polysaccharides (1185–900 cm^{-1}) and the fingerprint region (900–600 cm^{-1}) (Naumann et al., 1991). Some reports have been published using FTIR spectroscopy for rapid identification of species from *B. cereus group* (Mietke et al., 2010; Ehling-Schulz et al., 2005a), *B. subtilis* (Filip et al., 2004), *B. atrophaeus*, *B. circulans*, *B. lentus*, *B. thuringiensis* and *B. megaterium* (Brandes Ammann & Brandl, 2011; Forrester et al., 2009), however no reports were found on the differentiation within *B. pumilus* group species.

Despite the interest of several DNA-based methods for bacterial systematics, it is recommended a polyphasic approach resulting from the integration of genotypic, chemotaxonomic, phenotypic and ecological information for the construction of a reliable phylogenetic framework (Thompson et al., 2013).

3. Clinical Relevance, antimicrobial Resistance and Virulence within *Bacillus* spp.

Being *Bacillus* species widely used as feed additives for both humans and animals and deliberately introduced in the food chain, the European Food Safety Authority (EFSA) requires a status of Qualified Presumption of Safety (QPS) for these species (EFSA, 2007). This approach was created for the risk assessment of those microorganisms used in food/feed production, in order to avoid risk for consumers. Particular aspects that require attention in order to establish the safety of these microorganisms or their products in the industrial sector are the antimicrobial susceptibility profile and the virulence potential of a given species or taxonomic group (EFSA, 2012).

3.1 Clinical Relevance

B. cereus and closely related species are most often associated with human pathogenicity, where exotoxins, phospholipases, proteinases and haemolysins present a relevant role in the pathogenesis (Økstad & Kolstø, 2011). *B. anthracis*, the causative agent of anthrax, is responsible for the classic fatal systemic infection. The pathogenicity of *B. anthracis* is associated with the presence of two plasmids, pXO1 (coding form for the anthrax toxin) and pXO2 (coding for capsule formation) (Koehler, 2009).

Some *Bacillus* spp. are associated with opportunistic infections, e.g. in traumatic or post-surgical wounds, cancer patients or immunocompromised individuals (Wiedmann & Zhang, 2011). Infections of eyes, respiratory tract, central nervous system, septicemia, cases of fulminant necrotizing soft tissue and even endocarditis, have also been reported (Økstad & Kolstø, 2011; Bottone, 2010). In particular, species belonging to *B. pumilus* group have also occasionally been involved in human diseases (Kimouli et al., 2012; Johnson et al., 2008; Bentur et al., 2007; From et al., 2007a; Callegan et al., 2006; Haymore et al., 2006; Ozkocaman et al., 2006; Castagnola et al., 2001; Galanos et al., 2003; Peltola et al., 2001; Turnbull, 1997). In the hospital setting, *B. pumilus* group have been reported as causal agents of opportunistic infections mainly in chronically ill and immunosuppressed patients (Bentur et al., 2007; Haymore et al., 2006; Ozkocaman et al., 2006; Castagnola et al., 2001), in most cases associated with contaminated medicines, antiseptics and medical equipment, such as intravenous catheters or other medical devices (Bentur et al., 2007).

3.2. Antimicrobial Susceptibility of *Bacillus* spp.

The development of antibiotic resistance constitutes a serious concern, affecting a wide range of bacteria. Resistance to antimicrobial agents can be due to either (i) intrinsic properties (sometimes called 'natural resistance'), characteristic of all the strains of a certain species or by (ii) mutation of indigenous genes or the acquisition of resistance genes through mobile genetic elements, such as plasmids or transposons (Commission, 2002). Such intrinsic or acquired properties could make the bacteria capable of rapid inactivation of specific antibiotics through degradation, exportation of the antibiotics out of the cell, through the efflux system, or by alteration of the antibiotic target site (Butaye et al., 2003; Commission, 2002; Roberts et al., 1999). The presence of genes coding for antimicrobial resistance carried by mobile genetic elements, presents a greatest risk for horizontal dissemination of antimicrobial resistance between isolates from the same or even from different species (Devirgiliis et al., 2011; Reenen & Dicks, 2011).

According to the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), all bacterial products intended for use as feed additives must be examined to establish its susceptibility profile to a relevant range of antimicrobials of human and veterinary importance, e.g. ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol (EFSA, 2012). Specifically in

the case of *Bacillus* spp., EFSA determined as a basic requirement the determination of minimum inhibitory concentrations (MIC) and its interpretation according to microbiological cut-off values defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/) and the European monitoring program, as shown in Table 2.

Table 2. *Bacillus* spp microbiological cut-off values (µg/mL) for different antimicrobials according to FEEDAP panel (EFSA, 2012).

Antimicrobials	Microbiological cut-off value (µg/mL)
Vancomycin	4
Gentamicin	4
Kanamicin	8
Streptomycin	8
Erytromycin	4
Clindamycin	4
Tetracycline	8
Chloramphenicol	8

According to these guidelines, a bacteria presenting a MIC value higher than the cut-off value for a given antibiotic has an acquired resistance mechanism (EFSA, 2012), and according to the FEEDAP Panel when observed for one or more antimicrobials, it is recommended to explore the nature of resistance. The growing health concern about antimicrobial susceptibility profiles in *Bacillus* spp. is mainly due to the possible source of transfer of antibiotic resistance genes between bacteria (Gevers et al., 2003; Witte, 2000). Antibiotic resistance genes database (ARDB) (http://ardb.cbcb.umd.edu/) provides a centralized compendium of information on antibiotic resistance, providing a list of antibiotic resistance gene sequences identified in *Bacillus* spp. (Table 3).

Table 3. Antibiotic resistance genes and antibiotic resistance profiles identified in *Bacillus* spp. members enclosed in ARDB database.

Species	Antibiotic resistance gene	Antibiotic Resistance Profile	Acession number
B. anthracis str. sterne	*bacA*	Bacitracin[a]	YP_027565
	*bl2A*_1	Penicillin[b]	YP_029502
	fosB	Fosfomycin[c]	YP_030068
B. cereus str. E33L	*bacA*	Bacitracin	YP_082869 AAU18978 Q63DZ3
	fosB	Fosfomycin	Q63CC5 YP_083442 AAU18406
B. cereus str. Q1	*bacA*	Bacitracin	YP_002529178
	bl2A	Penicillin	YP_002530963
	fosB	Fosfomycin	YP_002529744
B. cereus str. B4264	*bacA*	Bacitracin	YP_002366165
	bl2A	Penicillin	YP_002368151 YP_002367214
	fosB	Fosfomycin	YP_002366749
B. thuringiensis	*bl2A*	Penicillin	Q45726
B. thuringiensis serovar *konkukian* str. 97-27	*bacA*	Bacitracin	AT61949 YP_035603
B. subtilis subsp. *subtilis* str. 168	*bl2A*	Penicillin	AAT60435 YP_037541
	fosB	Fosfomycin	YP_036189 AAT59669
	bacA	Bacitracin	ZP_03592903 NP_390993
	Blt	Chloramphenicol, doxorubicin,fluoroquinolone, puromycin[d]	NP_390536 AAC36944 ZP_03592438
	Bmr	Chloramphenicol, fluoroquinolone	AAB81539 ZP_03592168 NP_390281
	fosB	Fosfomycin	ZP_03591517 NP_389667
	lmrB	Lincomycin[e]	NP_388149 ZP_03589930
	tetL	Tetracycline[f]	ZP_03593907 NP_391957 BAA05208

	tmrB	Tunicamycin	NP_388196 ZP_03589973
B. subtilis subsp. *subtilis* str. NCIB 3610	*bacA*	Bacitracin	ZP_03597187
	Blt	Chloramphenicol, doxor ubicin, fluoroquinolone, puromycin	ZP_03596719
	Bmr	Chloramphenicol, fluoroquinolone	ZP_03596450
	fosB	Fosfomycin	ZP_03595797
	lmrB	Lincomycin	ZP_03594212
	tetL	Tetracycline	ZP_03598190
	tmrB	Tunicamycin	ZP_03594255
B. pumilus str. SAFR-032	*bacA*	Bacitracin	YP_001487997
B. pumilus	*catA*	Chloramphenicol	P00487
B. licheniformis	*bcrA*	Bacitracin	P42332 P42334
	bl2A	Penicillin	P00808
	ermD	Lincosamide, macrolide, streptogramin B[g]	Q03986 P45438
B. amyloliquefaciens str. FZB42	*bacA*	Bacitracin	YP_001422387 ABS75156
	fosB	Fosfomycin	YP_001420711 ABS73480
	lmrB	Lincomycin	ABS72697 YP_001419928
B. clausii str. KSM-K16	*bacA*	Bacitracin	BAD65785 YP_176746 Q5WCX5
B. halodurans str. C-125	*fosB*	Fosfomycin	NP_242644 BAB05497
B. circulans	*aph3iva*	Butirosin, kanamycin, neomycin, paromomycin, ribostamycin	CAA27061 P00553 0910185A
	vanA	teicoplanin, vancomycin[h]	CAB61227 CAB61228 CAB61229 CAB61230 CAB61231 CAB61224
	vanZ	Teicoplanin[i]	CAB61225

[a]Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.

[b]Class A beta-lactamase. This enzyme breaks the beta-lactam antibiotic ring open and deactivates the molecule's antibacterial properties.
[c]Glutathione transferase, metalloglutathione transferase which confers resistance to fosfomycin by catalyzing the addition of glutathione to fosfomycin.
[d]Major facilitator superfamily transporter. Multidrug resistance efflux pump.
[e]ABC transporter system, Macrolide-Lincosamide-Streptogramin B efflux pump.
[f]Major facilitator superfamily transporter, tetracycline efflux pump.
[g]rRNA adenine N-6-methyltransferase, which can methylate adenine at position 2058 of 23S rRNA, conferring resistance to erythromycin.
[h]VanA type vancomycin resistance operon genes, which can synthesize peptidoglycan with modified C-terminal D-Ala-D-Ala to D-alanine--D-lactate.
[i]VanZ confers low-level VanZ confers low-level resistance to the glycopeptide antibiotic teicoplanin.

Moreover, other multidrug resistance genes such as *cfr* (conferring resistance to five chemically unrelated antimicrobial classes, including phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A) have been identified in *Bacillus* species isolates, located in some of them in a plasmid carrying other resistance genes (Wang et al., 2012; Zhang et al., 2011; Dai et al., 2010). Therefore, the potential dissemination of these resistance genes among different bacterial species is worrisome and should be under surveillance.

3.3. Virulence potential of *Bacillus* spp.

3.3.1. *B. cereus* group

The first guidance to access virulence (toxigenic) potential of species of the *Bacillus* genus was developed by the Scientific Committee on Animal Nutrition (SCAN) and published in 2000 (Commission, 2000). In that document, it is assumed that toxins (enterotoxins) found in *Bacillus* species other than those of *Bacillus cereus* group would have sufficiently similar properties to be detected by the same detection methods. After 2000, few incidents of food poisoning were reported in non-pathogenic *Bacillus*-group, which were mostly related with the presence of heat-stable surfactins and similar cyclic lipopeptides. Examples of toxic peptides produced by *Bacillus* spp. comprise amylosin (*B. amyloliquefaciens*) (Mikkola et al., 2007), fengycin and surfactin from (*B. subtilis* and *B. mojavensis*) (Hwang et al., 2009; From et al., 2007a) or lichenysin (*B. licheniformis*) (Nieminen et al., 2007). Toxic effects have been detected only when they are produced in amounts able to cause demonstrable cell disruption, which is far from the typical concentration of its production and use (EFSA, 2013).

Pathogenicity potential is much more characterized for *B. cereus* group species than in other *Bacillus* species. The main enterotoxins associated with *B. cereus* virulence and that should be sought by diagnostic tools are the: i) non-hemolytic enterotoxin (Nhe, three-component toxin), ii) hemolysin BL (Hbl, three-component toxin), iii) and cytotoxin K (CytK, single-component toxin of the β-barrel pore-forming toxin family) (Stenfors Arnesen et al., 2008; Lund et al., 2000; Lund & Granum, 1996; Beecher & Macmillan, 1991) and iv) emetic toxin cereulide synthetase (Cereulide, cyclic heat stable depsipeptide) (Ehling-Schulz et al., 2006b; Ehling-Schulz et al., 2005b) (Table 4).

Table 4. *Bacillus* spp. toxins responsible for gastro-intestinal disorders. (Adapted from: Stenfors Arnesen et al., 2008).

Toxin	Gene	Nature	Gatrointestinal disorders infection/intoxication
Nhe	*nhe*	Protein, 3 components	Diarrheal
Hbl	*hbl*	Protein, 3 components	Diarrheal
CytK	*cytK*	Protein	Diarrheal
Cereulide	*ces*	Cyclic peptide, 1.2 kDa	Emetic

The protein *nhe* is the most commonly found enterotoxin gene complex, is probably ubiquitous in *B. cereus* group members and exhibits haemolytic activity towards erythrocytes from several mammalian species (Fagerlund et al., 2008) (Stenfors Arnesen et al., 2008). The *hbl* complex constitutes another pore-forming enterotoxin in the *B. cereus* group exhibiting also haemolytic activity towards erythrocytes from several animal species. (Wiedmann & Zhang, 2011). The *cytK* is a 34 kDa single-component protein toxin of the β-barrel pore-forming toxin family with necrotic, haemolytic, and enterotoxic effects (Lund et al., 2000). The emetic toxin (cereulide) is a small (1.2. kDa) non-ribosomally synthesized dodecadepsipeptide, which is heat stable, acid and protease resistant (Stenfors Arnesen et al., 2008). Its expression is affected by factors like oxygen, pH, and temperature and is regulated by the transitional state regulator AbrB (Lücking et al., 2009). Whereas the genes encoding the enterotoxins Nhe, Hbl and CytK are chromosomally encoded, the emetic toxin cereulide is encoded by a 208 kb plasmid, pCER270, with similarity to *B. anthracis* pXO1 and other pXO1-like plasmids (Rasko et al., 2007; Ehling-Schulz et al., 2006a; Hoton et al., 2005), highlighting the risk for lateral gene transfer between strains.

Several other candidate proteins have been suggested as potential contributors to the enterotoxigenic activity of *B. cereus,* for which more detailed information can be found in a review from Arnesen *et al*., (Stenfors Arnesen et al., 2008).

In the very few reports evaluating the cytotoxicity and production of putative emetic toxins in species other than those of the *B. cereus* group recovered from foods, water and food plants, the authors found only 8 out of 333 *Bacillus* strains tested to be putative toxin producers, particularly among *B. subtilis*, *B. mojavensis*, *B. pumilus*, or *B. fusiformis* isolates, but none of them produced *B. cereus*-like toxins (From et al., 2005). Although rare, the identification of toxins in non-*B. cereus* species from water, food, and food environments highlights the risk for food poisining and the need for further characterization of toxins and genes involved to improve detection methods (From et al., 2005).

It is also important to refer that the sole presence or absence of an individual toxin gene does not fully explain the pathogenicity or virulence potential of a certain strain, and consequently molecular methods used should always be accompanied by sensitive and accurate toxin quantification systems (Bauer et al., 2009).

3.3.2. *B. pumilus* group

Although rarely, some evidences of toxic properties of *Bacillus pumilus* have been reported, namely its cytopathic effects in Vero cells, haemolytic activity, lecithinase production, and proteolytic action on casein (From et al., 2005; Hoult & Tuxford, 1991). In addition, pumilacidin from *B. pumilus* was also reported as presenting toxic properties (From et al., 2007b) . Moreover, it also produces a toxin that has been detected in guinea pigs with experimentally induced enterocolitis associated with clindamycin (Knoop, 1979).

4. Biotechnological and industrial applications of *Bacillus pumilus* group members

Besides the high diversity characterizing *Bacillus* species at the taxonomic level, the diversity of their metabolic features is also noticeable (Novak et al., 2012; Gaggìa et al., 2010; Schallmey et al., 2004). Beyond their ability to produce a wide range of metabolites with very different natures and structures, they also display broad spectrum of activities,

including enzymes with great interest in detergent industry and food sectors; production of primary metabolites as vitamins (Schallmey et al., 2004) and ribonucleosides (Srivastava et al., 2012) and secondary metabolites comprising antibiotics, insecticides, biosurfactants agents and also growth promoting formulations (Lehman et al., 2013; Martinotti et al., 2013; Mulligan et al., 2014; Fracchia et al., 2012; Lehman et al., 2001; Schallmey et al., 2004), originally designed to enable the bacterium to survive in its natural environment (Stein, 2005).

The world market for industrial enzymes is estimated to be 1.6 billion $US, divided between food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%), for which is estimated that enzymes from *Bacillus* spp. constitute about 50% of the total of enzyme market (Berkeley et al., 2008). *B. pumilus* is involved in the alkaline serine proteases (subtilisins) production, with a reported primary application in household detergents, as Alcalase (Novo Nordisk) (Schallmey et al., 2004; Chen et al., 1995).

Among ribonucleosides, D-Ribose is frequently used as a flavor enhancer in food, pharmaceuticals, cosmetics, health food, and animal feed, as well as for the treatment of myocardiac ischemia and muscular pain, for which some *B. pumilus* are involved in its production (Srivastava et al., 2012).

In addition, poly-γ-glutamic acid (γ-PGA) is a water-soluble, eatable, and biodegradable compound with industrial applications in the food, cosmetics, and in medical fields and also in wastewater treatment as thickener, hemectant, cryoprotectant, drug carrier, highly water adsorbant hydrogel, biopolymer flocculent, heavy metal absorber, and animal feed additive (Shih & Van, 2001). Several *Bacillus* species, including *B. pumilus*, have been reported to produce γ-PGA, however, *B. licheniformis* and *B. subtilis* are the most widely studied species (Bhat et al., 2013).

The biosynthesis of several antibiotics and biosurfactant compounds is also noticeable from species belonging to *Bacillus* genus and *B. pumilus* group members in particular. Detailed information regarding theses aspects are given in Section III from this chapter.

Probiotic-containing products with *Bacillus* species are available for human nutrition, as animal feed supplements, and also for aquaculture (Hong et al., 2005; Rolfe, 2000; Verschuere et al., 2000)**,** with potential attributes as colonization, immunostimulation and antimicrobial activity. Originally, many commercial products were sold as products that carry *B. subtilis* spores, but recent studies have shown that most products are mislabeled and carry other *Bacillus* species, including *B. pumilus* (Green et al., 1999; Hoa et al.,

2000). In addition, other *B. pumilus* species were reported as antidiarrhoeal prophylactic agents by its probiotic activity (Sanders et al., 2003; Mazza, 1994).

Moreover, it is generally known that several microorganisms exhibit biological activity useful to control plant diseases. Indeed, *B. pumilus* is not considered an anti-insect pathogen like other members of the genus *Bacillus*, such as *B. thuringiensis* or *B. sphaericus* (Phelps & McKillip, 2002; Schirmer et al., 2002). Nevertheless, some reports identified this species as a biopesticide, with previous studies showing its activity against a broad range of phytopathogenic fungi (Lehman et al., 2013) and as an inducer of systemic acquired resistance (SAR) in plants (EFSA, 2013).

Regarding other species belonging to *B. pumilus* group, no evidences were found concerning its application for industrial or biotechnological purposes. Nevertheless, since appropriated identification methods of members of this group is a critical and still controversial step, which is being suffering considerable changes, it is unknown to which extent species form *B. pumilus* group are clearly discriminated in many of the available studies. Appropriated delineation of species and/or of clonal lineages adapted to particular niches will thus be of relevance to open new perspectives for biotechnological applications of *B. pumilus* group species.

II. Antimicrobial peptides of *Bacillus* spp.

Almost 90 years elapsed since Fleming discovered lysozyme, the first natural antimicrobial. Since then, more than 1200 types of antimicrobial peptides (AMPs) have been isolated from bacteria, animals, insects, and plants, and their use had a great impact in human health (Nakatsuji & Gallo, 2012)

The intensive and inappropriate use of antibiotic compounds has generated a strong selective pressure for the emergence of multi-drug resistant pathogens leading to a high percentage of morbidity and mortality rates (WHO, 2012), e.g. multidrug-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp. (VRE) (CDC, 2013). Moreover, nearly all of the antibiotics used today belong to classes discovered between 1941 and 1968 and it is particularly problematic that only two novel structural classes have become available on the market in the last 40 years: oxozolidiones and cyclic lipopetides (Calza et al., 2004; Jacqueline et al., 2004; Wagenlehner & Naber, 2004). More than ever, efforts are urgently needed to identify and develop new antibacterial compounds, more effective and with novel modes of action (Mills et al., 2011; Barsby et al., 2001).

In this sense, the screening of microbial natural products represents an important route to the discovery of novel chemicals, for development of new therapeutic agents and for evaluation of the potential of lesser-known and/or new bacterial taxa (Lazzarini et al., 2000). Therefore, exploration for new compounds from well-known and proficient microorganisms, such as *Bacillus*, due to the fact that only a small range of their biosynthetic capacity is currently exploited, has been conducted. In fact, recent advances in genome sequencing have highlighted the genus *Bacillus* as a source of antibiotic-like compounds resulting from their secondary pathways (Fickers, 2012).

5. Antimicrobial peptides (AMPs) types from *Bacillus* spp.

AMPs (AMPs, 2014) are natural products of metabolism that are not essential for normal growth, development or reproduction of an organism, generally comprise 10–50 amino-acid residues and are characterized by cationic amphipathic properties (Seo et al., 2012). In opposition to primary metabolites, these compounds are not ubiquitous in the living

organisms that produce them, neither are necessarily expressed continuously (Demain & Fang, 2000).

In fact, the function and importance of AMPs to the producer organism is usually related to ecological nature, as they are produced in nature for defense against predators, interspecies competition and to facilitate the reproductive processes contributing for the survival of the producers (Demain & Fang, 2000). Therefore, the ecological consequence of production of these compounds is the promotion and the maintenance of biodiversity in microbial communities through establishment of interaction networks.

Moreover, it seems that there is no relation between the presence of specific consensus amino-acid sequences associated and its biological activity, although most of them retain certain common chemical features as positive charge and relatively hydrophobic and amphipathic structures involved in their mechanism of action (Nakatsuji & Gallo, 2012; Lai & Gallo, 2009).

Generally, AMPs interact with microbial membranes, resulting in two possible modes of action, depending on the peptide-type and of microbial species (Nakatsuji & Gallo, 2012). The peptide can be membrane-disruptive resulting in cell lysis (Kjos et al., 2011; Diep et al., 2007; Cotter et al., 2005), or alternatively, membrane interaction which can lead to the formation of transient pores and to the transport of the peptides inside the cell, bringing them into contact with intracellular targets (Marlow et al., 2009; Mattiuzzo et al., 2007). Both mechanisms are related with peptide amphipathic features allowing them to interact with negatively charged phospholipid head groups and hydrophobic fatty acid chains of microbial membranes, resulting in pore formation and consequent release of cytosol components (Glaser et al.; Wimley, 2010).

The *Bacillus* genus produces distinct and heterogeneous AMPs which present different basic chemical structures (Gebhardt et al., 2002; Stein, 2005). These compounds can be subdivided into different classes, comprising: bacteriocins, bacteriocin-like inhibitory substances (BLIS), polyketides (PKs), nonribosomal peptide (NRPs), as well as other unusual and miscelaneous peptides.

In fact, *B. subtilis* produces more than two dozen of antibiotic compounds (Stein, 2005). If all pathways are considered, their production requires more than 350 kb, corresponding to a remarkable 10% of the annotated ORFs. It should also be emphasized that all known *B. subtilis* strains are able to produce individual antibiotic cocktails, which encompass only a portion of the compounds depicted (Stein, 2005). The potential of a given *B. subtilis* strain

for antibiotic syntheses is comparable with *B. amyloliquefaciens* (six operons of 340 kb, 8.5% of the genome) (Chen et al., 2009b). Moreover, while analyzing specific gene clusters and their functions, some researchers discovered additional AMPs gene clusters, leading to the hypothesis that most of the investigated microorganisms have a broader genetic capacity to produce natural products than the suggested by the number of compounds isolated from a particular strain (Gross, 2007).

5.1. Bacteriocins

Bacterial antimicrobial peptides of ribosomal synthesis that are lethal to bacteria, other than the producing strain, are commonly referred as bacteriocins (Riley & Wertz, 2002a; Riley & Wertz, 2002b; Vuyst & Vandamme, 1994). These heterologous groups of proteinaceous antimicrobial substances exhibit variable molecular weights and biochemical properties and also display a high degree of target specificity (Abriouel et al., 2011; Riley & Wertz, 2002a; Riley & Wertz, 2002b). Moreover, their potential use as natural preservatives in foods has been explored in the recent years (Abriouel et al., 2011; Deegan et al., 2006; Gálvez et al., 2008).

The first bacteriocin described was designated colicin and was produced by *Escherichia coli* (Cascales et al., 2007). Nevertheless, currently, the most studied bacteriocins are those produced by lactic acid bacteria (LAB), due to their applicability as biopreservatives in the food industry, and those produced by food chain important *Bacillus* species, including *B. subtilis* group members, which also possesses a history of safe use in industry (Pedersen et al., 2002).

Among *Bacillus* genus, *Bacillus* RNA group I members seem to be those with a greater significance concerning the production of these compounds, although other members of the remaining RNA groups also revealed the potential to produce them. Table 5 summarizes the bacteriocins described in the scientific literature among several *Bacillus* spp.

Table 5. Bacteriocins produced by *Bacillus* species.

***Bacillus* species**	**Source**	**Bacteriocin/BLIS**	**Molecular weight (kDa)**	**Special features**	**Class**	**Reference**
***Bacillus spp.* RNA group I**						
Bacillus subtilis group sensu lato						
B. subtilis	Culture collection	Subtilin	3.34	A-lantibiotic, binds lipid II	Subclass I.1	(Banerjee & Hansen, 1988; Stein, 2005)
	Culture collection	Subtilin B	3.42	Succinylated subtilin, A-lantibiotic, binds lipid II	Subclass I.1	(Chan et al., 1993)
	Chinese herbs, dairy product, soil, wild strains	Subtilosin A Subtilosin A1	3.393.41	Macrocyclic lantibiotic Macrocyclic lantibiotic, variant	Subclass I.4Subclass I.4	(Huang et al., 2009; Stein, 2005; Zheng et al., 1999)
	Culture collection	Mersacidin	1.82	Tetracyclic, B-lantibiotic, binds lipid II	Subclass I.2	(Bierbaum et al., 1995; Stein, 2005)
	–	Sublancin 168	3.88	AII-lantibiotic, unusual lantibiotic	Subclass I.2	(Paik et al., 1998; Stein, 2005)
	Fermented soybeans	Betacin	–	Produced by a SPβ lysogenic strain	–*	(Hemphill et al., 1980)
	–	MJP1	4.5	Antibacterial and antifungal activity	–*	(Yang & Chang, 2007)
	–	Ericin S	3.44	Lantibiotic; active against*Clavibacter*	Subclass I.1	(Stein et al., 2002)
	Plant rhizosphere	Ericin A	2.98	Lantibiotic	Subclass I.1	(Stein et al., 2002)
	Chinese herbs	Bac 14B	21	Antibacterial and antifungal activity	–*	(Hammami et al., 2009)
		LFB112	6.3	Activity against Gram-positive and Gram-negative bacteria	–*	(Xie J et al., 2009)
B. pumilus	Water	Pumilicin 4	1.99	Active against VRE and MRSA	–*	(Aunpad & Na-Bangchang, 2007)
B. licheniformis	Buffalo rumen	Lichenin	1.4	N-terminal: ISLEICXIFHDN	Subclass II.3	(Pattnaik et al., 2001)
	Dairy product	Bacillocin 490	2	–	–	(Martirani et al., 2002)
	Culture collections	Lichenicidin (α, β)	3.25 and 3.02	Two-peptide lantibiotic	Subclass I.3	(Begley et al., 2009)
	Soil	Peptide A-12 C	0.77	Antibacterial and antifungal activity	–*	(Gálvez et al., 1993)
B. amyloliquefaciens	Yogurt beverage	Subtilosin A	3.39	Macrocyclic bacteriocin from *B. subtilis*	–*	(BABASAKI et al., 1985)
Bacillus cereus group sensu lato						

B. cereus	Food	Cerein GN105	9	Active against other *B. cereus* strains	–*	(Naclerio et al., 1993)
	Soil	Cerein 7[a]	3.94	–	Subclass II.3	(Oscáriz & Pisabarro, 2000)
	Soil	Cerein 7B	4.89	*sec*-independent leader peptide with GG	Subclass II.3	(Oscáriz et al., 2006)
	Woodland	Cerein 8[a]	26	Aromatic amino acid residues	–*	(Bizani & Brandelli, 2002)
	Aquatic plant roots	Cerein MRX1	3.14	N-terminal: DWTCWSCLVCAACSVELL	Subclass II.2	(Sebei et al., 2007)
	Deep-surface oil reservoir	Cereicidin	–	Lantibiotic	–*	(Xiong et al., 2009)
B. anthracis	Culture collection	Heterocyclo-anthracins	9.62	Subfamily of putative thiazole-containing heterocyclic bacteriocins	–*	(Haft, 2009)
B. thuringiensis	Culture collection	Thuricin HD-2	>950	Phospholipase A activity	–*	(Favret & Yousten, 1989)
	Culture collection	Thuricin 439	2.92 and 2.80	Two singly active peptides	Subclass II.3	(Ahern et al., 2003)
	Soybean rot nodules	Thuricin 17	3.16	N-terminal: DWTXWSXL	Subclass II.2	(Gray et al., 2006a; Gray et al., 2006b)
	Honey	Thurincin H	3.14	Three structural genes, N-terminal DWTXWSXL	Subclass II.2	(Lee et al., 2009)
	Culture collection	Thuricin S	3.14	N-terminal: DWTXWSXL	Subclass II.2	(Chehimi et al., 2007)
	Soil litter	Bacthuricin F4	3.16	N-terminal: DWTXWSXL	Subclass II.2	(Kamoun et al., 2005)
	Human fecal sample	Thuricin CD	2.76 and 2.86	Dimer of two peptides; active against *C. difficile*	–*	(Hill et al., 2009)
	Culture collection	Entomocin 110	4.8	Active against *L. monocytogenes*	–*	(Cherif et al., 2008)
	Culture collection	Entomocin 9	12.4	–	–*	(Cherif et al., 2003)
	Soil	Thuricin 7	11.6	–	–*	(Cherif et al., 2001)
	Culture collection	Tochicin	10.5	–	–*	(Paik et al., 1997)
	Culture collection	Morricin 269	10	Wide inhibitory spectrum	–*	(Barboza-Corona et al., 2007)
	Culture collection	Kurstacin 287	10	Wide inhibitory spectrum	–*	(Barboza-Corona et al., 2007)
	Culture collection	Kenyacin 404	10	Wide inhibitory spectrum	–*	(Barboza-Corona et al., 2007)
	Culture collection	Entomocin 420	10	Wide inhibitory spectrum	–*	(Barboza-Corona et al., 2007)
	Culture collection	Tolworthcin 524	10	Wide inhibitory spectrum	–*	(Barboza-Corona et

						al., 2007)
Other *Bacillus spp.* belonging to RNA group I						
B. megaterium	Soil	Megacin A-216	32.85; 21.02, 11.85*	Phospholipase A activity	Class III	(Kiss et al., 2008)
	–	Megacin A-19213	39	Phospholipase A activity	Class III	(Von Tersch & Carlton, 1983)
	–	Megacin BII	–	Narrow spectrum	–*	(Stahl, 1989)
	Soil	Megacin C	150	Large protein	–*	(Donoghue, 1972)
	Soil	Megacin Cx	210	Large protein	–*	(Brusilow & Nelson, 1981)
	Vegetable wastes	Megacin 19	3.5–6.5	Broad spectrum against food-spoilage bacteria	–*	(Khalil et al., 2009b)
	Soil	Megacin 22	3.5–6.5	Broad spectrum against food-spoilage bacteria	–*	(Khalil et al., 2009a)
B. coagulans	Animal feces	Coagulin	4.6	Pediocin-like bacteriocin	Subclass II.1	(Le Marrec et al., 2000)
B. circulans	Poultry environment	SRCAM 1580	3.5	Pediocin-like bacteriocin; anti-*Campylobacter*	Subclass II.1	(Svetoch et al., 2005)
***Bacillus spp.* RNA group IV**						
B. brevis	Soil	Bacillocin Bb	<10	Broad spectrum, potential medical or food uses	–*	(Saleem et al., 2009)
	Wheat field	Brevicin AF01	–	Active against MRSA	–*	(Faheem et al., 2007)
	Mud/water samples	Thermoleovorin-N9	36	Broad spectrum	–*	(Novotny & Perry, 1992)
	Broiler chicken, crop	SRCAM 37	3.5	Pediocin-like bacteriocin; anti-*Campylobacter*	Subclass II.1	(Svetoch et al., 2005)
	Broiler chicken, intestine	SRCAM 602	3.5	Pediocin-like bacteriocin; anti-*Campylobacter*	Subclass II.1	(Svetoch et al., 2005)
	Kimchi	Paenibacillin	2.98	Lantibiotic	Subclass I.2	(He et al., 2007)
***Bacillus spp.* RNA group VI**						
B. atrophaeus	Culture collection	Subtilosin A	3.39	Macrocyclic bacteriocin from *B. subtilis*	–*	(Stein et al., 2004)
B. halodurans	Soil	Haloduracin (A1, A2)	3.04 and 2.33	Two-peptide-lantibiotic	Sunclass I.3	(McClerren et al., 2006)

*considered as BLIS

Recently, Abriouel et al. (Abriouel et al., 2011) proposed a specific scheme for *Bacillus* bacteriocins (Table 6), based on the main classification scheme for LAB ones.

Table 6. Proposed classification of bacteriocins of *Bacillus* species and comparison with ones from LAB. (Adapted from: Nes et al., 2007).

Proposed classification of *Bacillus* bacteriocins	**Bacteriocins examples**	**LAB bacteriocins correspondence**
Class I. Post-translationally modified peptides		Class I. Lantibiotics
Subclass I.1. Single-peptide, elongated lantibiotics	Subtilin Ericin S Ericin A	
Subclass I.2. Other single-peptide lantibiotics	Sublancin 168 Mersacidin	
Subclass I.3. Two-peptide lantibiotics	Haloduracin Lichenicidin	
Subclass I.4. Other post-translationally modified peptides	Subtilosin A	
Class II. Nonmodified peptides		Class II. Small, linear peptides
Subclass II.1. Pediocin-like peptides	Coagulin	Class IIa
Subclass II.2. Thuricin-like peptides	Thurincin H Thuricin S Thuricin 17 Bacthuricin F4 Cerein MRX1	
Subclass II.3. Other linear peptides	Cerein 7A Cerein 7B Lichenin Thuricin 439	
Class III. Large proteins	Megacin A-216 Megacin A-19213	Class III. Large heat-labile bacteriocins

Both class I and II bacteriocins are small (3–10 kDa), cationic, amphiphilic and membrane-active peptides (Lee & Kim, 2011). Moreover, class I includes antimicrobial peptides that undergo different types of post-translational modifications, essential for the antimicrobial activity of the final peptide (Abriouel et al., 2011). This class can be subdivided into four subclasses. Subclasses I.1–I.3 include peptides with modifications typical of lantibiotics (e.g. formation of lanthionine and β-methyl lanthionine residues), while subclass I.4 includes other unique modifications as particular sulfide bonds among

others. Subclass I.1 includes type A lantibiotics with an elongated structure, such as subtilin. Subclass I.2 includes the type B globular lantibiotic mersacidin, and other lantibiotics such as sublancin 168. Subclass I.3 includes the two-component lantibiotics. Subclass I.4 includes the unique cyclic peptide subtilosin A that contains a head-to-tail peptide bond as well as particular sulfide bridges formed between cysteine groups and dehydrated amino acid residues (Abriouel et al., 2011).

Moreover, the proposed classification seems to be coherent with clustering obtained from the comparative analysis of published amino acid sequences, as detailed in Figure 6. The subtilin and ericins form a coherent cluster, which is characterized by highly conserved regions in the mature peptide as well as in the leader peptide sequences. Interestingly, the A1 and A2 subunits of haloduracin and lichenicidin also form coherent clusters, suggesting that both two-peptide lantibiotics share a common origin. Mersacidin also shows homology with the A1 subunit of the two-peptide lantibiotics, and especially with the lichenicidin A1 subunit, with 25 conserved residues at identical positions, indicating its relatedness to the two-peptide lantibiotics (Abriouel et al., 2011).

Figure 6. Dendrogram showing the relatedness of *Bacillus* spp. bacteriocins according to primary amino acid sequence homologies. Shaded characters indicate identical amino acid residues. (Adapted from: Abriouel et al., 2011).

Bacteriocins of class II include small (0.77–10 kDa), non-modified and linear peptides, which are heat and pH stable (Table 6). This class can be subdivided into four

subclasses. Subclass II.1 includes pediocin-like peptides with a conserved YGNGVXC motif near the N-terminus (Figure 6), and the coagulin produced by *B. coagulans* I4. Subclass II.2 includes thuricin-like peptides with a conserved DWTXWSXL motif neighboring the N-terminus produced by *B. thuringiensis*, and cerein MRX1 produced by *B. cereus*. Comparative amino acid sequence analysis of thuricin-like peptides shows a coherent cluster (Figure 6). Subclass II.3 includes other linear peptides, such as lichenin produced by *B. licheniformis*, or cereins 7A and 7B.

Class III bacteriocins includes large proteins (430 kDa) with phospholipase activity such as megacins A-216 and A-19213 produced by *B. megaterium* (Table 6). Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by bacilli are not included in this classification scheme due to the lack of data on their protein or gene sequences.

Moreover, bacteriocin production and secretion seems to be correlated with bacterial stationary phase (Khalil et al., 2009a). Nevertheless, depending on the transcription regulation system, its expression may also be influenced by factors such as carbon sources (Drosinos et al., 2005), cell-density (Riley & Wertz, 2002b), temperature (Diep et al., 1994), or even the presence of a bacteriocin-sensitive strain (Barefoot et al., 1994).

Bacteriocins encoding genes are typically located in mobile genetic elements such as plasmids and transposons (Riley & Gillor, 2007) i. In fact, the production of a particular bacteriocin involves the co-expression of other genes that encode proteins associated with immunity and when required, secretion, regulation and biosynthesis (Jack et al., 1995; Nes et al., 1996). Thus, bacteriocins and the associated genes are usually expressed in an operon, but may involve expression of up to four separate operons (Heng et al., 2006).

Some of specific aspects of most common bacteriocins produced from *Bacillus* spp., namely subtilin, ericin, sublancin, mersacidin, subtilosin A, haloduracin and lichenicidin are detailed and compared in Figure 7 a), b) and c).

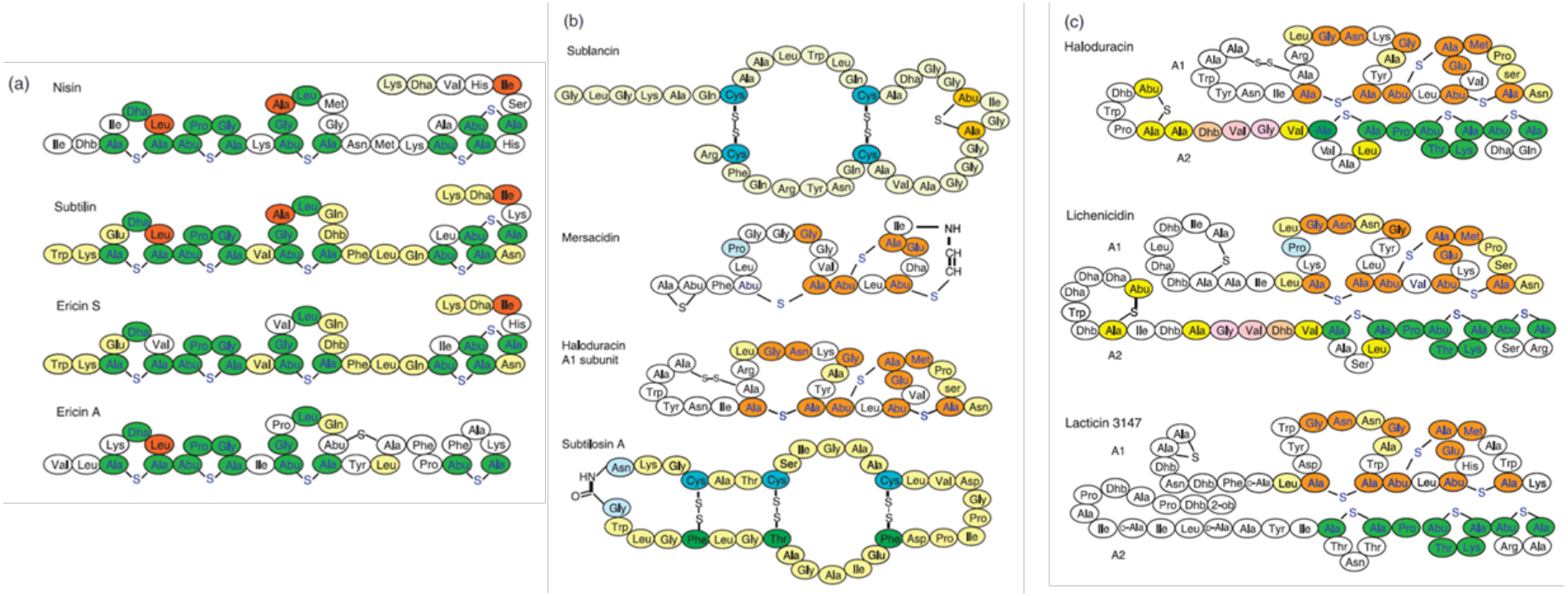


Figure 7. (a) Comparison of subtilin and ericin structures with that of the lactococcal lantibiotic nisin A. Conserved residues at identical positions to all four bacteriocins are highlighted in green, while those conserved only in subtilin and ericins are depicted in yellow; other conserved residues are in light red. (b) Structures of the single-peptide lantibiotics sublancin, mersacidin and subtilosin A. The structure of the A1 subunit of haloduracin is also included for comparison with mersacidin. The conserved residues are highlighted in orange color. Cysteines involved in disulfide bridge formation are highlighted in blue. For subtilosin A, residues involved in sulfur to a-carbon linkages are shown in green, while residues involved in head-to-tail amide bond formation are in light blue. (c) Comparison of the two-peptide lantibiotics haloduracin and lichenicidin from *Bacillus*, and lacticin 3147 from *Lactococcus lactis*. Conserved residues of the A1 peptides are highlighted in orange and light yellow. A conserved Pro residue between lichenicidin A1 subunit and mersacidin is shown in light blue. Conserved residues of the A2 peptides are highlighted in green, deep yellow, light orange and

violet. The C-terminal parts of the A1 subunits also share two conserved loops of 11 and eight amino acid residues. The C-terminal parts of A2 peptides share the same pattern of lanthionine (Ala–S–Ala) and methyllanthionine (Abu–S–Ala) bridges. Non-identical residues are in white. (Adapted from: Abriouel et al., 2011).

Concerning bacteriocins commercially available, unfortunately, only Nisin is currently approved by the US Food and Drug Administration for application as a natural preservative in food. Mersacidin is a compound with remarkably interest. It inhibits the synthesis of *S. aureus* cell wall, also demonstrating activity against MRSA, with a similar efficiency of vancomycin (Jenssen et al., 2006). Therefore, since its mode of action differs from vancomycin, a possible combination of mersacidin with this antibiotic could be a promising alternative.

5.2. Bacteriocin-like inhibitory substances (BLIS)

The term BLIS is often used when the peptide nature of the antimicrobial compound has not been confirmed, whereby ribosomal synthesis is presumed, but may be unknown (Abriouel et al., 2011). Main features of the described BLIS from different *Bacillus* species are presented in Table 5.

Different BLIS with distinct spectra of activity against both Gram-positive and Gram-negative bacteria were described in *B. subtilis* (Stein et al., 2004; Stein et al., 2002; Stein, 2005; Paik et al., 1998; Bierbaum et al., 1995). Pumilicin was reported in *B. pumilus* and is a plasmid-encoded peptide (Aunpad & Na-Bangchang, 2007; Lovett et al., 1976). This compound presents a molecular weight of 1994.62 Da, heat stability up to 121 ºC, is active in a pH range of 3–9 and demonstrates remarkable antibacterial activity against MRSA, vancomycin-resistant *E. faecalis* (VRE) and several Gram-positive test bacteria (Aunpad & Na-Bangchang, 2007).

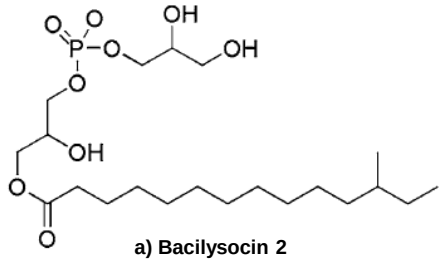
5.3. Unusual peptides

Bacillus spp. are able to synthetize other unusual antibiotic peptides, such as rhizocticins and phosphonate oligopeptide which contain C-terminal nonproteinogenic amino acid (Z)-l-2-Amino-5-Phosphono-3-Pentenoic Acid (APPA) (Borisova et al., 2010; Kino, 2010; Kino et al., 2009). These molecules are synthesized by the called L-amino acid ligase, which is able to catalyze the formation of an alpha-peptide bond from L-amino acids in an ATP-dependent manner (Tabata et al., 2005).

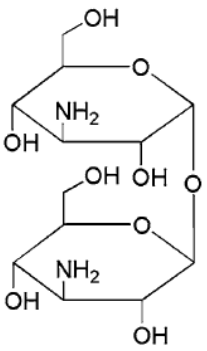
Rhizocticin A is able to prevent the growth of yeast and filamentous fungi by protein synthesis inhibition but is not active against bacteria (Borisova et al., 2010; Kino et al., 2009; Diddens et al., 1979; Laber et al., 1994). Furthermore, some strains of *B. subtilis* produce the dipeptide bacilysin composed of L-alanine and the unusual amino acid L-anticapsin (Walker & Abraham, 1970).

5.4. Other antibiotic compounds (miscellaneous)

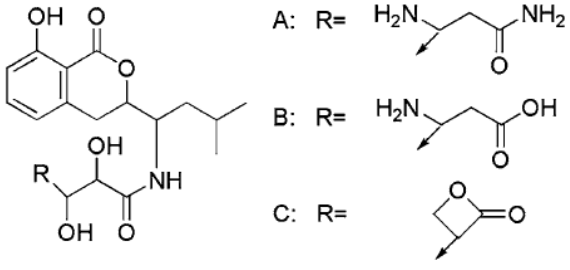
Miscellaneous antibiotics comprise a class of compounds that are structurally different from the remaining AMPs families and from each other and for this reason cannot be classified into the other classes. Examples of representatives agents produced by *B. subtilis* include bacilysocin 2 (Tamehiro et al., 2002), 3,3´-neotrehalosadiamine (NTD) 3 (Inaoka & Ochi, 2007) and amicoumacin 4 (Pinchuk et al., 2002) (Figure 8).



a) Bacilysocin 2



b) 3,3´-Neotrehalosadiamine (NTD) 3



c) Amicoumacin 4

Figure 8. Chemical structures of miscellaneous antibiotics bacilysocin 2, 3,3´-neotrehalosadiamine (NTD) 3 and amicoumacin 4. (Adapted from: Sansinenea & Ortiz, 2011).

Bacilysocin 2 (Figure 8a) is an antimicrobial phospholipid derived from the major *B. subtilis* phospholipid phosphatidylglycerol through YtpA-catalysed acyl ester hydrolysis (Tamehiro et al., 2002).

NTD 3 (Figure 8b), structurally 3,3´- diamino-3,3´-dideoxy-a,b-trehalose (Tsuno et al., 1986), inhibits *S. aureus* and *Klebsiella pneumoniae* and functions as an auto inducer and also as a glucose uptake modulator in *B. subtilis* by activating its own biosynthetic operon (Inaoka & Ochi, 2007).

Amicoumacins 4 (Figure 8c) present antibacterial and anti-inflammatory activities, namely in *Helicobacter pylori*, which make these compounds attractive for the treatment of chronic gastritis and peptic ulcer in humans (Pinchuk et al., 2002).

5.5. Polyketides and non-ribosomal peptides

Polyketides (PKs) are a highly active class of secondary metabolites produced by almost all living organisms, currently used in human and animal health and in agriculture as antibiotic (erythromycin, rifamycin), antifungal, immunosuppressant and antitumor agents (Li et al., 2011; Nakano et al., 2009; Resmi & Soniya, 2012). Structurally, they encompass organic compounds exhibiting remarkable diversity in terms of their structure, which can be divided into three classes, type I, II and III (Chen et al., 2006). Some PKs from *B. subtilis* and also fungi and plants, are grouped in the third class (Resmi & Soniya, 2012; Li et al., 2011; Nakano et al., 2009).

Concerning PKs produced by *Bacillus* spp., they are synthesized on modularly organized assembly lines by elongation of activated monomers of amino and hydroxyl acid building blocks following the same logic as NRPs (Hertweck et al., 2007).

Difficidin, macrolactin and bacillaene are three examples of PKs isolated from *B. amyloliquefaciens* (Arguelles-Arias et al., 2009; Chen et al., 2007) (Figure 9).

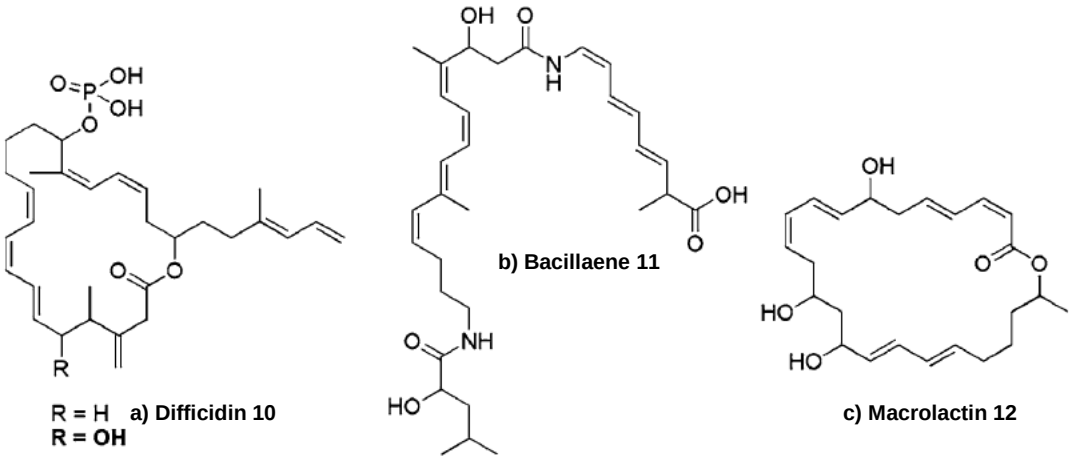


Figure 9. Chemical structures of polyketides antibiotics a) difficidin, b) bacillaene and c) macrolactin (Adapted from: Sansinenea & Ortiz, 2011).

Difficidin (Figure 9a) is an unsaturated 22-membered macrocylic polyene lactone phosphate ester with a broad spectrum of antibacterial activity. It inhibits protein biosynthesis and was recently used as a grow suppressive agent against *Erwinia amylovara*, a devasting plant pathogen causing necrotrophic fire blight disease of apple, pear and other rosaceous plants (Chen et al., 2009b).

Bacillaene (Figure 9b) and macrolactin (Figure 9c) are antimicrobial agents that could be potentially useful in human medicine. Both are prokaryotic protein synthesis inhibitors which display antimicrobial activity toward human pathogens such as *Serratia marcescens*, *K. pneumonia* and S. aureus (Chen et al., 2009a; Patel et al., 1995). Macrolactin consists of a 24-membered ring lactone which has the ability to inhibit murine melanoma cancer cells as well as mammalian herpes simplex viruses, and also showed effective activity in protecting lymphoblast cells from HIV (Gustafson et al., 1989). Moreover, 17 macrolactins have been described and one of them, 7-O-malonyl macrolactin A, was effective against Gram-positive bacterial pathogens (Romero-Tabarez et al., 2006).

5.6. Non-ribosomal peptides (NRPs)

Currently, NRPS comprehend hundreds of molecules, which can be found in Norine database (Caboche et al., 2008).

Since the discovery of the linear polypeptide produced by *B. brevis* named gramicidin (Kapoerchan et al., 2012), in the early 1970's, (Figure 10a), used primarily as a topical antibiotic against Gram-positive bacteria, many other bioactive NRPs have been isolated and their biosynthetic clusters characterized.

A NRPS widely used is Bacitracin (Figure 10b) which is composed by a mixture of related cyclic polypeptides produced by *B. licheniformis* and related counterparts and is used in human medicine, inhibiting the biosynthesis of the bacterial cell wall by interacting with undecaprenyl pyrophosphate, involved in peptidoglycan synthesis (Stone & Strominger, 1971). This antibiotic was primarily used in topical formulations against Gram positive bacteria namely *Streptococcus* spp and *S. aureus* (Awais et al 2008). Recently, 2010, injectable bacitracin was approved by US FDA for the treatment of infants with staphylococcal pneumonia and empyema (Sagent, 2010).

Polymyxin B (Figure 10c), a cyclic cationic lipopeptide produced by *B. polymyxa*, was introduced in 1960 due to the rise of MDR Gram negative bacteria, and is now frequently the last resort for several infections (Kwa et al., 2008).

Finally, daptomycin (Figure 10d), a lipopeptide antibiotic, active against Gram-positive bacteria involved in skin and soft tissue infections, is currently the most frequent bactericidal drug on the market and is used as a reserve antibiotic against MDR Gram positive cocci like methicillin-resistant *S. aureus* (MRSA and vancomycin-resistant enterococci (VRE) (Steenbergen et al., 2005).

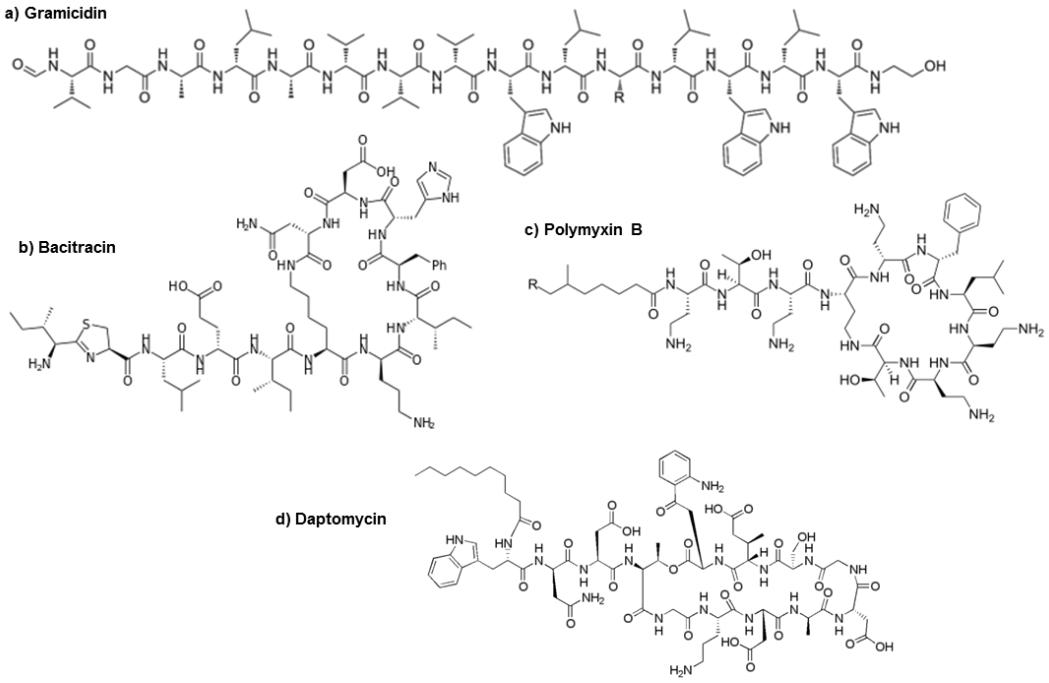


Figure 10. Chemical structures of polypeptide antibiotics a) gramicidin, b) bacitracin, c) polymyxin B and d) daptomycin.

Moreover, other interestingly molecules enclosed in this class, namely lipopeptide compounds, will be detailed in section III of this chapter.

III. Biosurfactants production within *Bacillus* spp.

6. General features of *Bacillus* spp. biosurfactants

Biosurfactants are amphipathic molecules of microbial origin with both hydrophilic and hydrophobic (generally hydrocarbon) moieties, which partition preferentially at the interface between fluid phases with different degrees of polarity, such as oil/water or air/water interfaces (Desai & Banat, 1997). They include low molecular-weight compounds, such as lipopeptides, glycolipids and proteins, and high-molecular-weight polysaccharides, lipopolysaccharides, proteins or lipoproteins (Mulligan, 2005), which exhibit pronounced tensioactive properties, namely surface and emulsifying activities. Moreover, these compounds are considered relatively nontoxic, biodegradable and effective at extreme temperatures or pH values, but perhaps more importantly, present a unique chemical structure with a high structural diversity (Martinotti et al., 2013).

In fact, they are usually produced as complex mixtures of up to 40 analogues, among which the hydrophilic head group are fairly conserved and the hydrophobic tail group present considerable variation among the families (Soberón-Chávez, 2010). In contrast to common synthetic surfactants that typically possess alkyl chains of ten or more carbon units, many natural biosurfactants possess short alkyl chains enhancing their aqueous solubility by means of relatively weak Van der Waals attractive interactions. Biosurfactants generate aggregates in solution and consequently, exhibit powerful surfactant activity at both liquid and solid phases. Despite their aqueous solubility, they can have remarkably low critical micelle concentration (CMC) when compared to structurally similar synthetic surfactants (Martinotti et al., 2013).

Nevertheless, the main commercialized surfactants are synthetic molecules, although its high cost of production constrains their use on a large scale. So far, only biosurfactants belonging to rhamnolipids classes are commercially available for industrial use, since they are the only compouds that had been approved by US Environmental Protection Agency for use in food products, cosmetics, and pharmaceuticals (Nitschke & Costa, 2007).

Although most biosurfactants are considered as bacterial secondary metabolites, some of them play essential roles for the survival of the microorganisms that produce them, either

through facilitating nutrient transport, microbe-host interactions or as biocide agents. In general, biosurfactant roles include the increase of the bioavailability of hydrophobic water-insoluble substrates, heavy metal binding capacity, bacterial pathogenesis, quorum sensing and biofilm formation (Singh & Cameotra, 2004b).

As a result of their remarkable properties, there is a wide interest in these molecules for diverse applications, such as bioremediation of organics (Mulligan, 2009; Chen et al., 2005; Shin et al., 2005; Uysal & Turkman, 2005; Urum & Pekdemir, 2004; Garcia-Junco et al., 2003; Schippers et al., 2000) and metals (Wang & Mulligan, 2009; Dahrazma & Mulligan, 2007; Neilson et al., 2003; Ron & Rosenberg, 2002; Mulligan et al., 2001a), cosmetic additives (Soberón-Chávez, 2010; Yoneda et al., 2001), pharmaceutical preparations (Fracchia et al., 2012; Singh & Cameotra, 2004b) and also as coatings agents (Rodrigues & Teixeira, 2010; Meylheuc et al., 2006; Rodrigues et al., 2006a; Rodrigues et al., 2006b; Rodrigues et al., 2004).

Nevertheless and despite recent developments in the optimization of production processes and in its engineering, many of the potential applications depend on whether they can be cost effective. The possibility of production of biosurfactants from inexpensive waste substrates, thereby decreasing their production costs, has also been reported (Sen, 2010). Additionally, legal aspects such as stricter regulations concerning the environmental pollution by industrial activities will strongly influence the chances of biodegradable biosurfactants to replace their chemical counterparts (Sen, 2010).

6.1. Biosurfactants basic structure and classification

In general, a biosurfactant consists in lipophilic and hydroplilic portions. The lipophilic moiety consists usually on hydrocarbon (alkyl) tails of one or more fatty acids, which may be saturated, unsaturated, hydroxylated or branched (Cameotra et al., 2010). Fatty acid are linked to the hydrophilic moiety (carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol) by a glycosidic ester or amide bond in Figure 11.

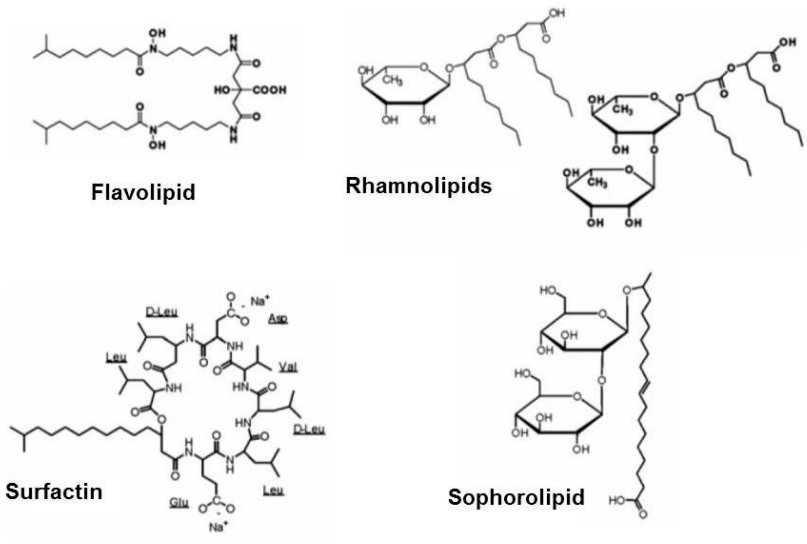


Figure 11. Representative structures of different biosurfactants.

The physicochemical characteristic that defines a biosurfactant is its ability to increase the apparent water solubility of hydrophobic compounds, to form water hydrocarbon emulsions, and to reduce surface tension (Desai & Banat, 1997), which is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface (Soberón-Chávez, 2010). Effective biosurfactants lower the surface tension between water and air from 72 to 35 mN/m and the interfacial tension between water and n-hexadecane from 40 to 1 mN/m. When surfactant monomers are added to the solution, the surface or interfacial tension will decrease until the surfactant concentration reaches what is known as the Critical Micelle Concentration (CMC) (Figure 12), which is dependent on the molecule structure, pH, ionic strength, and temperature of the solution (Soberón-Chávez, 2010).

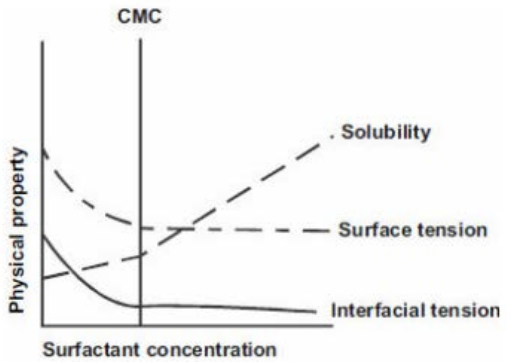


Figure 12. Relationship of surface tension, interfacial tension and CMC related to surfactant concentration. (Adapted from: Mulligan, 2005).

Above the CMC no further reduction in surface or interfacial tension is expected and, at the CMC, surfactant monomers begin to spontaneously associate into structured aggregates such as micelles, vesicles, and lamellae (continuous bilayers). These aggregates are created as a result of numerous weak chemical interactions between the polar head groups and the nonpolar tail groups including hydrophobic, Van der Waals, and hydrogen bonding, and its structure is dependent on the solvent polarity in which the surfactant is dissolved (Soberón-Chávez, 2010).

On the basis of their molecular mass, biosurfactants are generally divided into two groups: (i) low molecular weight compounds, including lipopeptides and glycolipids and (ii) high molecular weight agents, including lipoproteins, proteins, polysaccharides, lipopolysaccharides, phospholipids, fatty acids, neutral lipids or complexes (e.g. polymeric and particulate compounds) (Mulligan, 2005).

Low molecular weight biosurfactants comprise compounds that are more effective in lowering surface tension and interfacial tension. They usually appear as a mixture of related compounds with major components ranging in size from 979 to 1091 Da (Fracchia et al., 2012). Moreover, glycolipids are the most common class of known biosurfactants consisting in mono or disaccharides compounds (carbohydrates) in combination with acylated long-chain aliphatic acids or hydroxyaliphatic acids. Among them, rhamnolipids, mannosylerythritol lipids (MELs), sophorolipids and trehalolipids are the best-studied structural subclasses (Fracchia et al., 2012).

High molecular weight biosurfactants, also known as bioemulsifiers, are generally effective stabilizers of oil-in-water emulsions, and thus, possess effective emulsifying activity. The heteropolyssaccharide backbone contains a repeating trisaccharide of N-acetyl-D-galactosamine, N-acetylgalactosamine uronic acid, and an unidentified N-acetyl amino sugar and fatty acids are covalently linked to the polysaccharide through *o*-ester linkages (Fracchia et al., 2012).

6.2. Biosurfactants diversity

Little is known about the distribution and frequency of biosurfactants produced by *Bacillus* spp. in natural ecossystems. Several *Bacillus* species isolated from soil- and plant-associated environments and also deep sea sediments (Raaijmakers et al., 2010), injection brine (Raaijmakers et al 2010), fermented food (Mukherjee & Das, 2005) and

human gastrointestinal tract (Hong et al., 2009) revealed the ability to produce lipopeptides biosurfactants (LPBSs), althought their function in these ecological niches remains poorly explored.

Additionally, biosynthesis of some LPBSs seems to be correlated with some *Bacillus* species (e.g. iturins and *B. subtilis*, *B. amyloliquefaciens* and *B. pumilus* or surfactin and fengycin which are widespread among multiple species) (Kim et al., 2009; Pueyo et al., 2009; Snook et al., 2009; Li et al., 2008; Huszcza & Burczyk, 2006; Kim et al., 2004; Kalinovskaya et al., 2002; Tsuge et al., 1999), or even to the geographic origin of the strains (Price et al., 2007). The authors speculate that this species-specificity allows the *Bacillus* populations to compete better with the microbial communities indigenous to each particular habitat, and also provide an an adequate response to changes in abiotic and biotic conditions in their natural habitats (Price et al., 2007).

However, stability and persistence of LPBSs in complex environments have also not yet been extensively studied.

6.3. Lipopeptides Biosurfactants (LPBSs): a high diversity of structures

Most of LPBSs produced by *Bacillus* spp. are classified into different families encompassing surfactins (Arima et al., 1968), iturins (Delcambe et al., 1977), fengycins (Vanittanakom et al., 1986), kurstakins (Hathout et al., 2000), bacillomycins (Roongsawang et al., 2010) and mycosubtilin (Duitman et al., 1999) (Table 7). In addition, other lipopeptides have also been identified in *Bacillus* species, although they have not yet been assigned to a family.

Table 7: Primary structure and fatty acid chains of representative LPBSs families produced by *Bacillus* spp.

Name	Primary structure of the peptide moiety	Main fatty acid chains	References
Surfactin family	**Heptapeptide closed by a lactone ring with the β -OH group of the fatty acid chain**	**β-OH fatty acids**	
Surfactin	L-Glu-L-XS_2-D-Leu-L-XS4-L-Asp-D-Leu-L-XS_7	*i*C_{14}, *n*C_{14} *i*C_{15}, *a*C_{15}	Peypoux et al., 1999
Lichenysin	L-XL_1-L-XL_2-D-Leu-L-XL_4-L-Asp-D-Leu-L-XL_7	*i*C_{13}, *a*C_{13}, *n*C_{13} *i*C_{15}, *a*C_{15}	Grangemard et al., 1999; Lin et al., 1994; Lee et al., 2007
Bamylocin A	Glu-Leu-Met-Leu-Pro-Leu-Leu-Leu	C_{13}	
Esperin	L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-XE_7-COOH	C_{13}, C_{14}, C_{15}	Thomas & Ito, 1969
Iturin family	**Heptapeptide closed by a lactam ring with the β -NH_2 group of the acid chain**	**β-NH_2 fatty acids**	
Bacillomycin D	L-Asn-D-Tyr-D-Asn-L-Pro-L-Glu-D-Ser-L-Thr	*n*C_{14}, *i*C_{15}, *a*C_{15}	Peypoux et al., 1981
Bacillomycin F	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Thr	*n*C_{16}, *i*C_{17}, *a*C_{17}	Peypoux et al., 1985
Bacillomycin L or Lc or Bacillopeptin	L-Asn-D-Tyr-D-Asn-L-Ser-L-Glu-D-Ser-L-Thr	*n*C_{14}, *i*C_{15}, *a*C_{15}	Volpon et al., 2007
Iturin A	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*C_{14}, *i*C_{15}, *a*C_{15}	Peypoux et al., 1978; Isogai et al., 1982
Iturin A_L	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*C_{16}, *i*C_{16}	Winkelmann et al., 1983
Iturin C	L-Asp-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*C_{14}, *i*C_{15}, *a*C_{15}	Peypoux et al., 1986; Volpon et al., 2007
Mycosubtilin	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn	*n*C_{16}, *i*C_{16}, *a*C_{17}	Peypoux et al., 1986; Duitman et al., 1999
Fengycin family	**Decapeptide with a lactone ring between carboxy-terminal group of Ile10 and OH group of Tyr3**	**β-OH fatty acids**	

Fengycin A	L-Glu-D-Orn-D-Tyr-D-a Thr-L-Glu-D-Ala-L-Pro-L-Gln-L-Tyr-L-Ile	*a*C_{15}, *i*C_{16}, *n*C_{16}	Volpon et al., 2000; Schneider et al., 1999
Fengycin B	L-Glu-D-Orn-D-Tyr-D-a Thr-L-Glu-D-Val-L-Pro-L-Gln-L-Tyr-L-Ile	*a*C_{15}, *i*C_{16}, *n*C_{16}, C_{17}	Volpon et al., 2000; Schneider et al.,1999
Plipastatin A	L-Glu-D-Orn-D-Tyr-D-a Thr-L-Glu-D-Ala-L-Pro-L-Gln-D-Tyr-L-Ile	*n*C_{16}, *a*C_{17}	Nishikiori et al., 1986; Volpon et al. 2000
Plipastatin B	L-Glu-D-Orn-D-Tyr-D-a Thr-L-Glu-D-Val-L-Pro-L-Gln-L-Tyr-L-Ile	*n*C_{16}, *a*C_{17}	Nishikiori et al., 1986
Kurstakin family	**Heptapeptide with a lactone ring between carboxy-terminal group of Gln_7 and OH group of Ser_4**	**β-OH fatty acid chain**	
Kurstakin	D-Thr-Gly-D-Ala-Ser-His-D-Gln-Gln	*i*C_{11}, *n*C_{12}, *i*C_{12}, *i*C_{13}	Hathout et al., 2000

Each LPBSs family comprises several variants, which can differ in their fatty acid chain and their peptide moiety (Figure 13). Moreover, the existence of several different compounds with the same molecular weight emphazises the need to a precise characterization of their chemical structure.

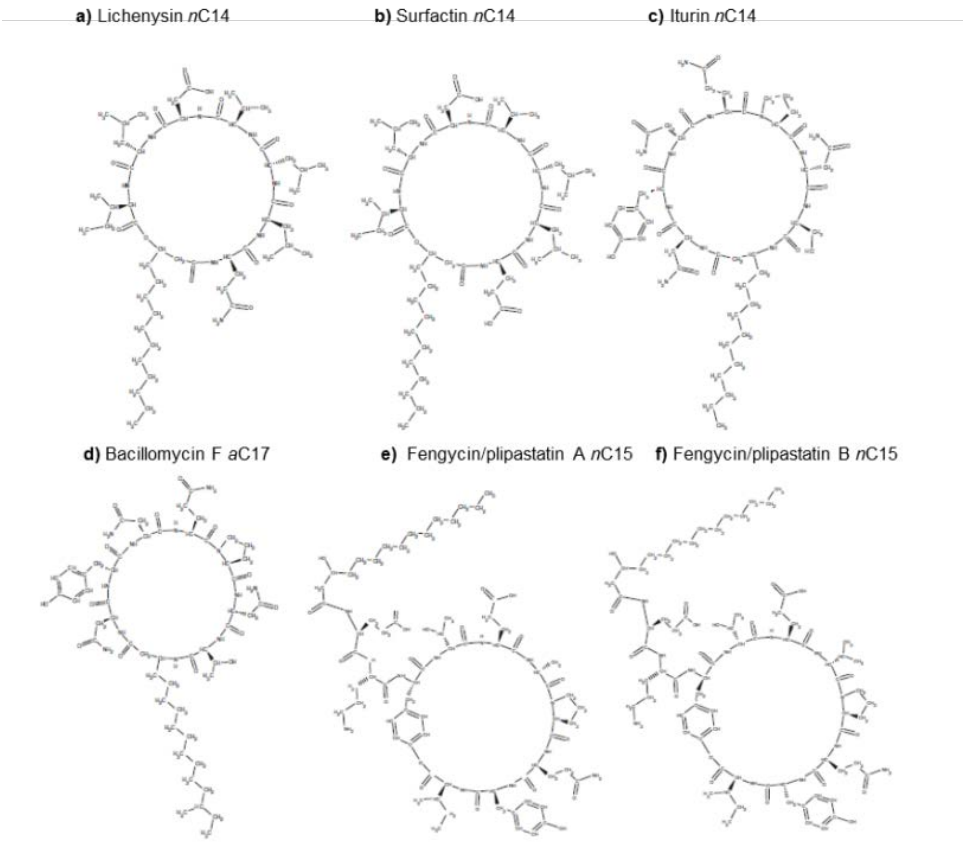


Figure 13. Detailed structure of some representative biosurfactants produced by *Bacillus* spp.

6.3.1. Surfactin family

The family of surfactin encompasses about 20 different LPBSs (also named surfactin-like compounds) (Bonmatin et al., 2003). With exception of esperin (Thomas & Ito, 1969), all of them present a common structural trait, which include a heptapeptide with a chiral sequence “LLDLLDL” interlinked with a β-hydroxy fatty acid (C_{14} to C_{17}) and with a D-Leu in position 3 and 6 and L-Asp in position 4. Amino acid residues present in position 2, 4 and 7 belong to the aliphatic group, and include Val, Leu and Ile (Bonmatin et al., 1995; Itokawa et al., 1994; Peypoux et al., 1991).

Moreover, LPBSs are usually a mixture of compounds with different lengths and types of fatty acid (FA), β-hydroxy FA (FA-β-OH), β-amino FA (FA-β-NH_2) or guanidylated-β-OH FA (*g*FA-β-OH), in which the β-OH or β-NH_2 group of FA forms an ester or a peptide bond with the carboxyl group of the *C*-terminal amino acid. The presence of these variants is a result of the biosynthesis mechanism of these compounds that involve non-ribosomal peptide synthetases (NRPSs), a process detailed in sub-section 6.4.

In the literature it is established that lipopeptides which present a Glu in position 1 are named surfactin, while those that possess a Gln are named lichenysin, since it was discovered from a *B. licheniformis* isolate (Horowitz et al., 1990). This surfactin-like compound is at least 2-fold more efficient than surfactin, reducing the surface tension of water from 72 to 27 mN/m at a CMC of 25–220 mg/L depending on its variants and determined conditions (Grangemard et al., 1999; Arima et al., 1968). Moreover, the occurrence of an Asn in position 4 was first mentioned in the structure described for lichenysin A by Yakimov et al. (Yakimov et al., 1995). In addition, other surfactin-like compound, constituted by Leu4, Val7 or Ile7 surfactin, was isolated from a *B. pumilus* and was named pumilacidin (Morikawa et al., 1992).

Esperin differs from the surfactin by a lactone ring involving the β-carboxyl of Asp in position 4, instead of the α-carboxyl of the terminal Leu. The β-hydroxy fatty acid chain linked to these different peptide moieties can contain 12 - 16 C atoms and exhibit n, iso and anteiso configurations. The main fatty acid chains are usually C_{14} and C_{15} (Soberón-Chávez, 2010).

Bamylocin A, a recently described lipopeptide, was isolated from *B. amyloliquefaciens* (Lee et al., 2007). The peptide chain of this molecule is Glu-Leu-Met-Leu-Pro-Leu-Leu-Leu and the molecular weight of the C_{13} form differs by less than 0.1 mass unit from the standard surfactin C_{14} isoform. This new result demonstrates the need of a precise mass spectrometry analysis of surfactin molecules to precisely confirm their primary structure.

For fengycin, circulocins, fusaricidin, and kurstakin, the carboxyl group of the *C*-terminal amino acid is lactonised with the hydroxyl group of Tyr_3, Thr_1, Thr_1, and Ser_4, respectively (Roongsawang et al., 2010).

6.3.2. Iturin family

Iturin A, the best well studied lipopeptide from the iturin family, is a heptapeptide interlinked with a β-amino acid fatty acid, that present a length from C_{14} to C_{17} (Peypoux et al., 1978). Six other members of the iturin family were also recognized, which include iturin C, bacillomycin D, F, L and Lc and also mycosubtilin (Bonmatin et al., 2003). Generally, all iturins present the same "LDDLLDL" chiral sequence with a common part in the peptidic portion, β-amino acid L-Asx-D-Tyr-D-Asn (Soberón-Chávez, 2010). With the exception of Iturin C, the first amino acid of the peptide chain is L-Asn. Moreover, Volpon et al. (Volpon et al., 2007) confirmed the presence of L-Asn in position 1 of bacillomycin L instead of L-Asp as initially described, which demonstrates that bacillomycin Lc and bacillomycin L present the same structure. In opposition to some surfactin variants, members of the iturin family probably result from different synthetases. Additionally, the main length of the fatty acid chain differs on its members, of which FA of iturin A, iturin C, bacillomycin D and L presented a length of C_{14}, C_{16} (Winkelmann et al., 1983) and C_{15}, while C_{16} and C_{17} are representatives of bacillomycin F and mycosubtilin (Soberón-Chávez, 2010).

6.3.3. Fengycin family

This family includes lipodecapeptides (as fengycins), which differ by their amino acid residue in position 6: Ala (present in form A) or Val (form B) (Soberón-Chávez, 2010). They present an internal lactone ring in the peptidic moiety between the carboxyl terminal amino acid (Ile), and the hydroxyl group, in the side chain of the tyrosine residue (position 3). Different β-hydroxy fatty acid chains (C_{14} to C_{18}) can be linked with an amide bond to the N-terminal amino acid residue (Glu) (Nishikiori et al., 1986; Vanittanakom et al., 1986), although representative fatty acid chains are C_{15}, C_{16} and C_{17}. With the exception of a single lipopeptide isolated from the supernatant of *B. thuringiensis*, which present a fatty acid chain with one double bond between carbons 13 and 14 (Kim et al., 2004), all of them are saturated (Soberón-Chávez, 2010).

Two differences were initially identified in fengycin and plipastatin molecules, which included a Gln instead of a Glu in position 8, and the L and D forms of tyrosine, which are presented in position 3 and 9, respectively, for plipastatins and 9 and 3 for fengycins (Soberón-Chávez, 2010).

6.3.4. Other Lipopeptide Compounds: Kurstakins

Kurstakins are a novel class of lipopeptides which include several lipoheptapeptide with the same amino acid sequence: Thr-Gly-Ala-Ser-His-Gln-Gln (Hathout et al., 2000). Different fatty acyl chains (isoC_{11}, nC_{12}, isoC_{12} and isoC_{13}) are linked by an amide bond to the N-terminal amino acid residue. Moreover, each lipopeptide has a lactone linkage between the carboxyl terminal amino acid and the hydroxyl group on the side chain of the serine residue. The L and D forms of the amino acid residues are not yet characterized. However, the recent identification of the genes involved in the biosynthesis of such or similar compounds indicated that amino acids in positions 1 and 6 could be in presented D-form (Bumpus et al., 2009; Abderrahmani et al., 2011).

6.4. Biosynthesis of LPBSs: from genes to biomolecules

Non-ribosomal peptide synthetases (NRPSs) are responsible for the biosynthesis of most LPBSs from *Bacillus* spp. by a thiotemplate process although polyketide synthetases (PKS) as well as fatty acid synthetases are also involved (Soberón-Chávez, 2010). Moreover, analysis of metabolic profiles of *Bacillus* species shows that single strains can simultaneously produce representatives of different lipopeptide familes, but also multiple structural analogues of one particular lipopeptide.

6.4.1. The non-ribosomal peptide synthesis machinery

Non-ribosomal peptide synthesis involves large multienzymatic proteins organized in modules, named non-ribosomal peptide synthetases (NRPSs), which are multi-modular enzymes that recognize, activate, modify and link the amino acid intermediates to the product peptide (Koglin & Walsh, 2009; Sieber & Marahiel, 2005). Additionally, they are capable of synthesizing peptides that incorporate unusual amino acids, including D-, β- and hydroxy- or *N*-methylated amino acids.

a) Primary catalytic domains

The catalytic domains within the NRPSs modules can be classified into several different types. The four main types of domains include the adenylation (A), the thiolation (T) or peptidyl carrier protein (PCP), the condensation (C), and the thioesterase (TE), which

together comprise the minimal set of domains required for a fully working NRPS, creating a linear assembly (Figure 14).

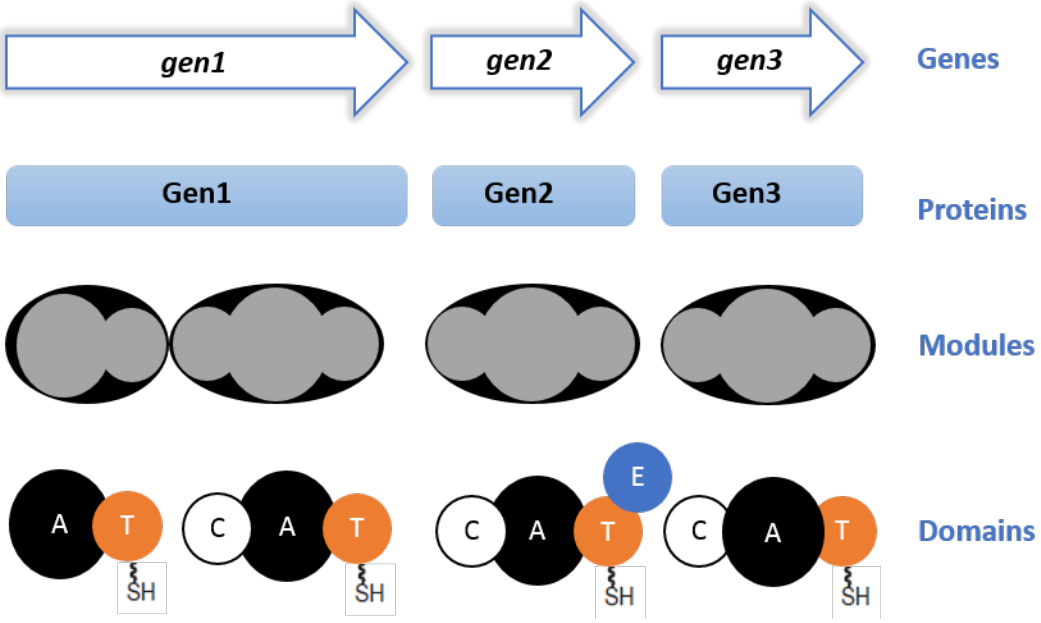


Figure 14. Overview of NRPS relationship between genes, modules and domains. Each gene is related with a single protein, which can then be prepared through one of more modules. These modules are organized into domains, represented here by colors (A, adenylation domain; C, condensation domain; PCP, peptidyl carrier protein and E, epimerisation domain). (Adapted from: Jenke-Kodama & Dittmann, 2009).

The Adenylation (A) domain is responsible for the amino acid recognition and ATP-dependent activation of an amino acid to form an acyl-adenylate intermediate. Then, the adenylated amino acid binds covalently to a phosphopantetheine carrier of the adjacent thiolation (T) or peptidyl carrier protein (PCP) domain. In addition, peptide bond formation of two consecutively amino acids is catalysed by the condensation (C) domain and modification domains such as the epimerization (E) catalyse the conversion of L-amino acids to D-isomers. Finally, cyclization and release of the product peptide are carried out by *C*-terminal thioesterase (Te) domain, which is associated with a termination module (Roongsawang et al., 2010). An example of this structured multi-modular complex is shown in Figures 14, 15 and 16.

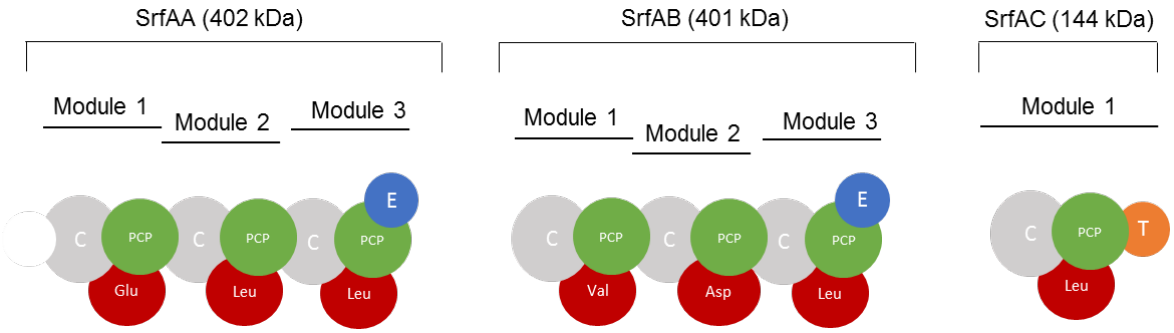


Figure 15. General representation of biosynthesis and structure of surfactin (*srf)* operon. Assembly line of surfactin biosynthesis with condensation domains are colored in gray, adenylation domains in red and peptidyl carrier proteins in green. Epimerization domains are shown in blue, the thioesterase domain in orange. (Adapted from: Kraas et al., 2010).

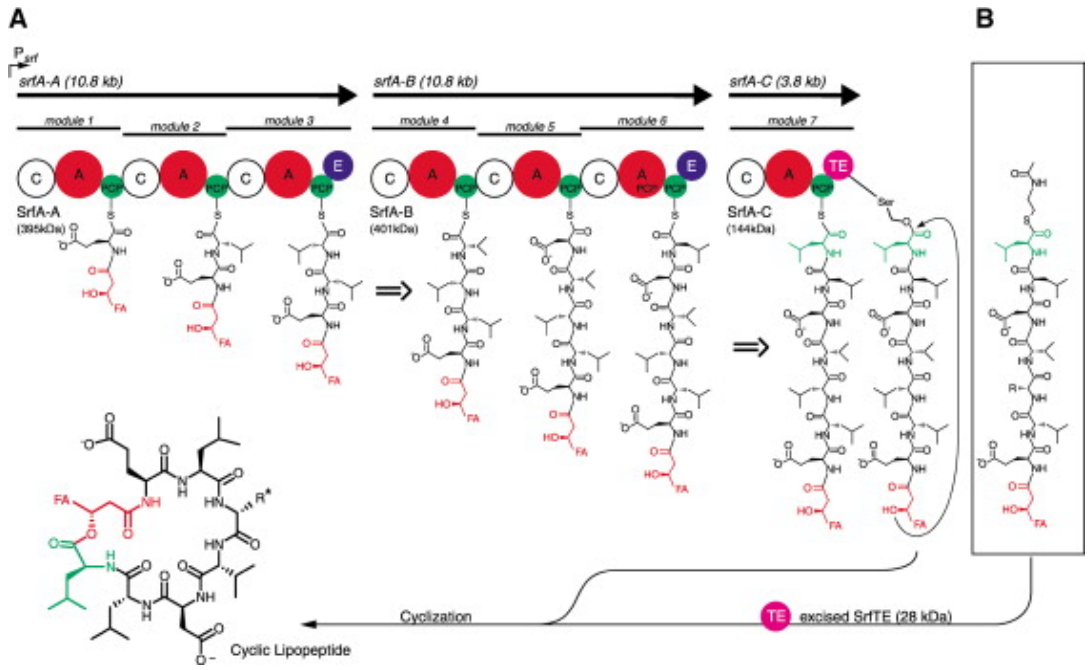


Figure 16. Surfactin biosynthesis by the Modular Peptide Synthetase. (A) The *srf* operon (top) presente three genes *srfA-A*, *srfA-B*, and *srfA-C*, which coded for surfactin synthetase subunits, shown below the genes. Bars indicate the module position within the protein, whereas the individual domains are shown as colored balls: A, adenylation domain; PCP, peptidyl carrier protein domain; C, condensation domain; E, epimerization domain; TE, thioesterase domain. 4′-phosphopantetheinyl cofactors with active thiol groups were shown with the corresponding peptides attached at their current synthesis states. The growing peptide chain is passed from left to right, until the linear product at the last PCP domain is cyclized to the lipopeptide by the TE domain. (B) The SNAC (S-N-

acetyl cysteamine) acyl peptide can be cyclized by the genetically excised SrfTE domain. The native peptide (R = DLeu) and the soluble substrate (R = DOrn) are illustrated. (Adapted from: Bruner et al., 2002)

b) Secondary catalytic domains

A second thioesterase domain can be also encoded within the NRPS gene cluster, and can be called type II thioesterases (TEII). It presents only 10% of sequence identity with the type I thioesterases and can be involved in the regeneration of carrier proteins that have been mis-acylated. In fact, as A-domains are specific to a substrate, there is a chance, depending on the selectivity of the A-domain, to load the wrong amino acid onto the carrier protein. If this occurs, as had been shown within the gene clusters of some NRPS such as surfactin (TEIIsr f) and bacitracin (TEIIbac), TEII are responsible for the hydrolysis of the misacylated thiol groups of the 4´-phosphopantetheine arm (Schwarzer et al., 2002).

6.4.2. Diversity of NRPSs in *Bacillus* spp.

Several gene clusters encoding NRPS for the biosynthesis of LPBSs in *Bacillus* spp., have been cloned and characterized (Figure 17). They present similarities in the modular architecture of their repetitive catalytic units and assembly-line mechanism, although some distinct unique features can be recognized. Characteristics of these LPBSs families and corresponding NRPSs are detailed in subsequent sections.

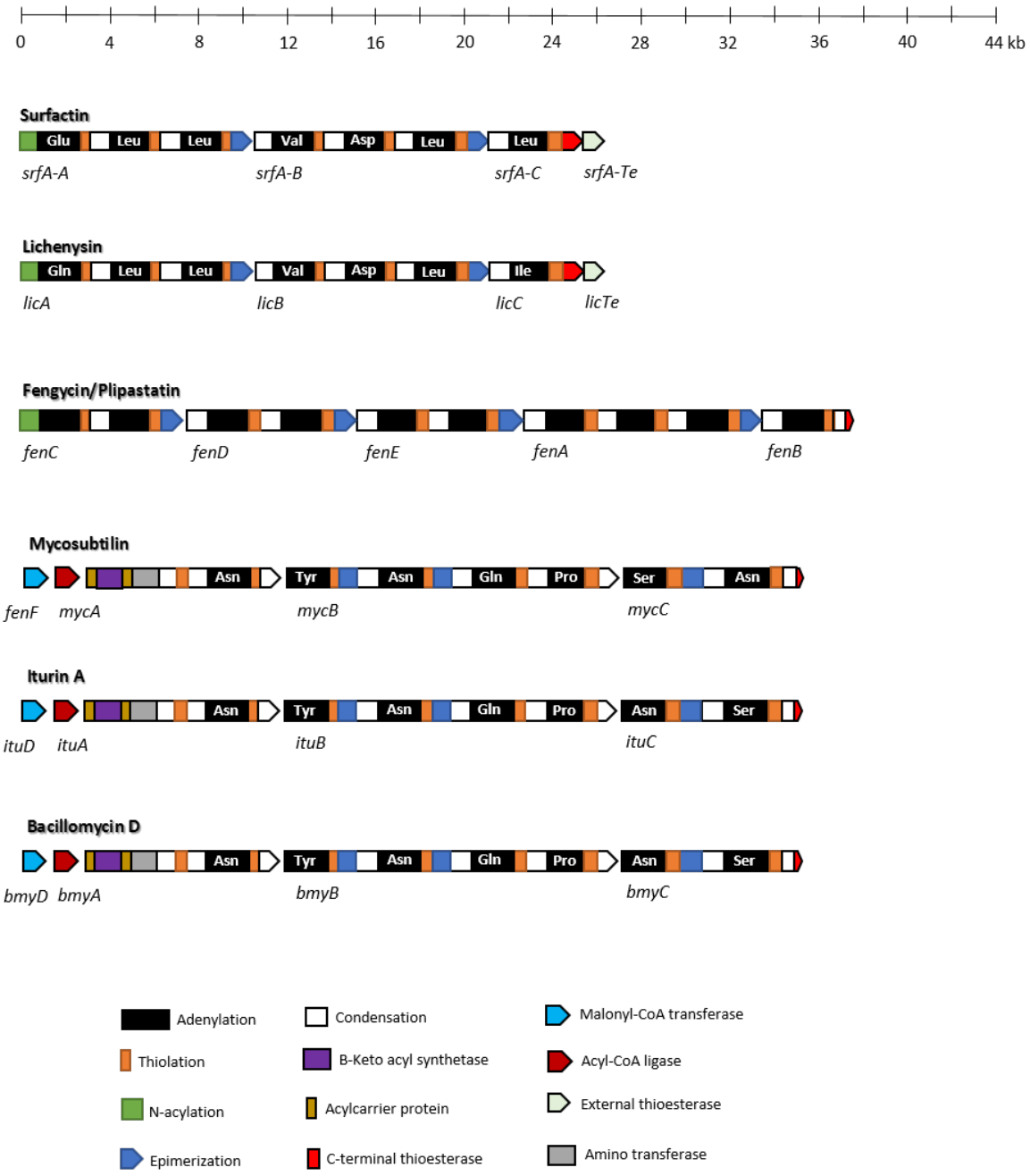


Figure 17. Multidomain organization of the representative gene clusters encoding NRPSs in *Bacillus* spp. genes encompassing each peptide synthetase operon, including their sizes and organization within the modules. Surfactin, liquenysin, fengycin/plipastatin, mycosubtilin, Iturin A and bacillomycin D are represented. (Adapted from: Roongsawang et al., 2010).

6.4.2.1. Surfactin and Lichenysin Synthetases

The surfactin (Cosmina et al., 1993) and lichenysin (Konz et al., 1999) biosynthetic gene clusters, particularly *srfA* and *lic*, are highly homologous and presented 25 kb (Fig. 7). They enclose four open reading frames (ORFs), *srfA-A*/*licA*, *srfA-B*/*licB*, *srfA-C*/*licC*, and *srfA-Te*/*lic-Te*. Moreover, amino acid sequences of the first three ORFs are homologous to other NRPSs, whereas the last ORF encodes a putative type II-Te. *SrfA* and *Lic* carry six typical C-domains that catalyse amide bond formation. *SrfA* and *Lic* encompass the conventional E-domains essential for the transformation of L-amino acids into D-amino acids and also a *C*-terminal type I Te-domain that releases the final product. In addition to the *C*-terminal type I Te-domain, SrfA and Lic have an external type II Te protein, SrfA-Te/LicTe. This protein is involved in regenerating misprimed T-domains by removing short acyl chains from the 4′-phosphopantetheine cofactors and thereby regenerates functional NRPSs (Schwarzer et al., 2002). Moreover, (Yeh et al., 2004) suggested that the type II Te also hydrolyzes incorrectly loaded amino acids, which are not processed by the nonribosomal machinery (Yeh et al., 2004). Additionally, SrfA-Te also functions as the thioesterase/acyltransferase that supports and stimulates the formation of β-hydroxymyristoyl-glutamate, an initiation substrate of surfactin synthesis (Steller et al., 2004; Menkhaus et al., 1993).

6.4.2.2. Fengycin Synthetase

Fengycin, is also referred as plipastatin when Tyr is present in positions 3 and 9, as the L- and D-form, respectively. Fengycin forms a lactone between the hydroxyl group of L-Tyr_3 and C-terminal carboxyl group of L-Ile. Fengycin synthetase (*Fen*) contains five NRPS subunits, namely, *fenC* (287 kDa), *fenD* (290 kDa), *fenE* (286 kDa), *fenA* (406 kDa), and *fenB* (146 kDa), which are ordered accordingly (FenC-FenD-FenE-FenA-FenB). Like SrfA and Lic, Fen is also composed of an *N*-acyl domain at the *N*-terminus of *fenC*, commonly E-domains, and also presents a typical type I Te-domain (Leclère et al., 2006) (Figure 17).

6.4.2.3. Iturin, Bacillomycin and Mycosubtilin Synthetases

Iturin family encompasses the cyclic lipoheptapeptides iturin, bacillomycin, and mycosubtilin. NRPS gene cluster of bacillomycin D (*bam/bmy*), mycosubtilin (*myc*), and

iturin A (*itu*) are composed by four large ORFs (Figure 17) (Koumoutsi et al., 2004; Moyne et al., 2004; Duitman et al., 1999; Tsuge et al., 2001a).

bam and *bmy* present identical gene clusters, which encode multifunctional hybrid enzymes of a fatty acid synthase, an aminotransferase and a peptide synthetases. Moreover, the first ORF-*bmyD*, *ituD*, and *fenF* encodes a malonyl-CoA transacylase, and the second ORF-*bmyA*, *ituA*, and *mycA* encode the acyl-CoA ligase, an acyl carrier protein (ACP), a β-ketoacyl synthetase and an aminotransferase domain, before the conventional module of NRPS (Aron et al., 2007; Hansen et al., 2007; Aron et al., 2005).

Mycosubtilin and iturin A present the same structure except that D-Ser_6 and L-Asn_7 residues in mycosubtilin molecules are replaced by D-Asn_6 and L-Ser_7 in iturin A (Tsuge et al., 2001a).

6.4.3. Gene Regulation in *Bacillus* spp.

Two component systems and quorum sensing play an important role in gene regulation of LPBSs produced by *Bacillus* spp. (Duitman et al., 2007; Sullivan, 1998). Among all, the surfactin biosynthesis system is the most extensively studied. The expression of surfactin genes is associated with increased cell density and occurs mainly in the transition from exponential to stationary growth phase, whereas in the expression of fengycins and iturin genes occurs usually later in the stationary phase (Mulligan et al., 2014).

Generally, in the surfactin biosynthesis system, the expression of the gene *srfA* is controlled by several peptide pheromones, which include ComA/ComP, the cell-density dependent pheromone ComX, and the phosphatase RapC (Magnuson et al., 1994).

Bacillus subtilis encodes eight Phr peptides (PhrA, PhrC [CSF], PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) also referred as the competence stimulating factors, and 11 aspartyl-phosphate phosphatase proteins (RapA to RapK). Each Phr peptide inhibits the activity of the co-transcribed Rap protein. Moreover, RapC, RapF and RapK act as negative regulators of *srfA* (Auchtung et al., 2006) and ComX interacts with the membrane-bound histidine kinase ComP, which auto-phosphorylates upon stimulation and consequently transfers its phosphate to a serine residue in the response regulator ComA. The phosphorylated ComA binds to promoter region of *srfA*, particularly in ComA boxes (T/GCGG-N4-CCGCA) located upstream of the *srfA* promoter and thus initiates transcription of *srfA* (Yakimov & Golyshin, 1997; Roggiani & Dubnau, 1993).

The complexity of the regulatory network is further exemplified by the observation that expression of the *srf* genes is also modulated by other transcription factors such as DegU (Mäder et al., 2002) or the H_2O_2 stress-responsive PerR (Hayashi et al., 2005), both acting as positive regulators. Additionally, overexpression of RapD, RapG, and RapH inhibits *srfA* transcription, and production of these Rap proteins is suppressed by RghR (Ogura & Fujita, 2007; Hayashi et al., 2006). Mutation in *sodA*, which encodes superoxide dismutase, inhibits transcription of the comQXP quorum-sensing locus, thereby preventing *srfA* expression (Ohsawa et al., 2006). Moreover, at high concentrations of the amino acids Ile, Leu, and Val, CodY represses transcription of *srfA* byinteracting specifically with the *srfA* promoter (Serror & Sonenshein, 1996). Similar to CodY, AbrB also negatively regulates *srfA* transcription. Expression of *srfA* is also repressed by the RNA polymerase-binding protein Spx (Nakano et al., 2003). On the other hand, the Spx-RNA polymerase interaction is required for positive transcriptional control of genes in response to thiol-oxidative stress (Reyes & Zuber, 2008).

Furthermore, 4′-phosphopantetheinyl transferase (Sfp/PPTase) encoded by the *sfp* gene located downstream of srfABCD (Steller et al., 2004) is required for the activation of SrfA enzymes by converting the inactive *apo*-forms of the T-domains to the active *holo*-forms (Quadri et al., 1998). An acyltransferase SrfA-Te is also required in the initial step of transferring a hydroxyl fatty acid to the first amino acid in the peptide. Finally, the surfactin self-resistance protein, YerP, is required for surfactin exportation (Tsuge et al., 2001b). An overview of this mechanism is shown in Figure 18.

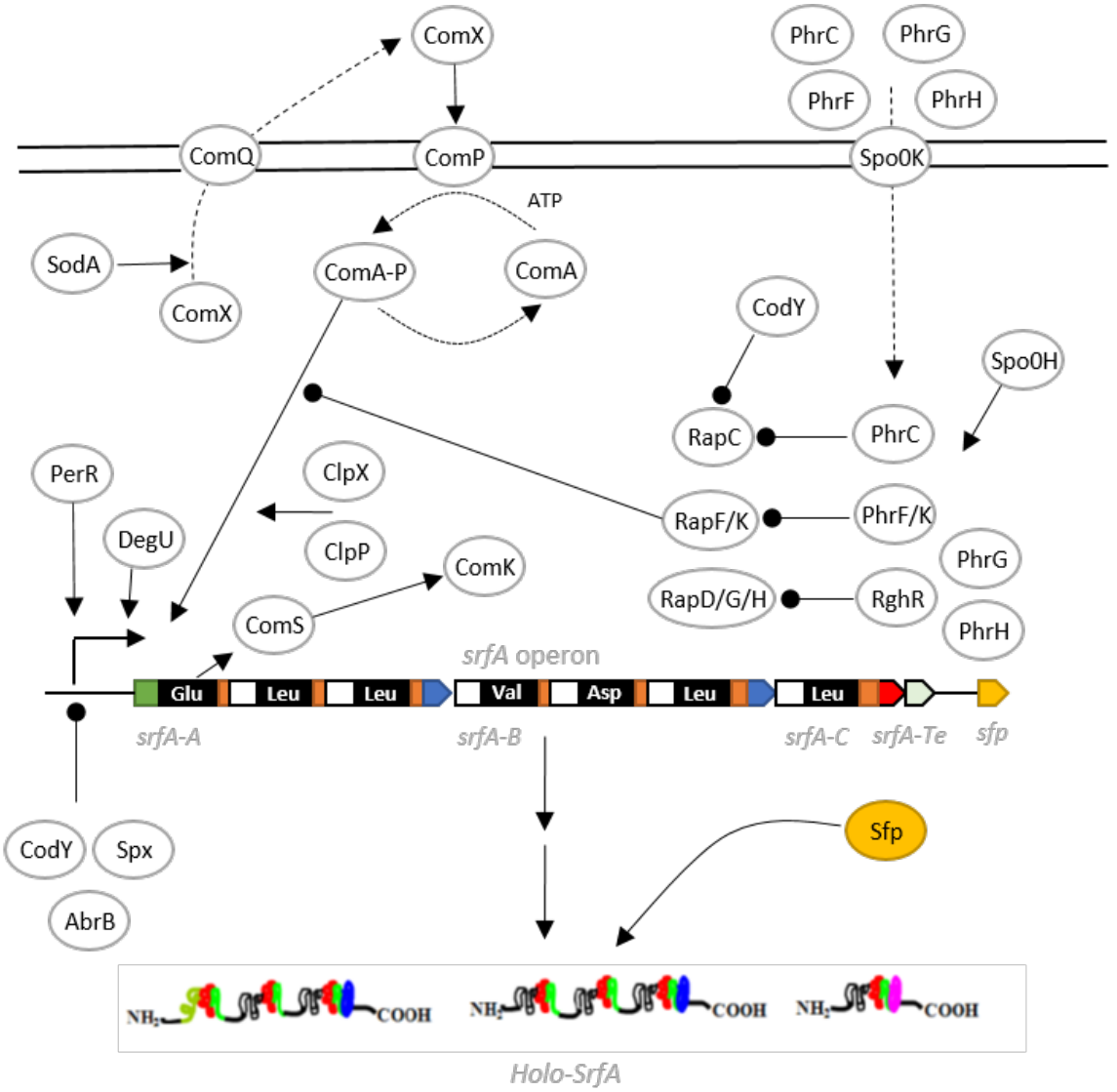


Figure 18. Overall model of gene regulation involved in surfactin biosynthesis. Closed-head arrows indicate positive regulation whereas closed circles indicate negative regulation. (Adapted from: Roongsawang et al., 2010).

The regulation of fengycin/plipastatin genes is positively controlled by DegQ, an enhancer of extracellular protease production (Tsuge et al., 1999) in which, *degQ* is the regulatory gene that controls the production of several hydrolytic enzymes (Msadek et al., 1991). Moreover, a Sfp-like protein, Lpa-8, is also required for plipastatin production in *B. subtilis* YB8 (Tsuge et al., 1996).

Gene regulation of the iturin family was first demonstrated within mycosubtilin-producing *B. subtilis* ATCC6633. Expression of the *myc* operon is independent of ComA, but still appears to be regulated via quorum sensing, since PhrC strongly stimulates expression. Moreover, sigma H factor, Spo0H, also influences expression of the *myc* operon. Transition state regulator AbrB represses expression of *myc*, since deletion of *abrB* culminate in the increased of *myc* expression (Duitman et al., 2007).

6.5. Natural functions of LPBSs

LPBSs influence the ecological fitness of the procucer bacterial strain, being important for their establishment in a specific environment (Ron and Rosenberg 2001). Therefore, the main physiological role of biosurfactants is to support the growth of microorganisms on water-immiscible substrates by reducing the surface tension at the phase boundary making the substrate more readily available for uptake and metabolism.

6.5.1. Antagonism

It has been proposed that, in natural habitats, lipopeptides may confer a competitive advantage in interactions with other microorganisms. When tested *in vitro*, biosurfactants produced by *Bacillus* spp., exhibit lytic and growth-inhibitory activities against a broad range of microorganisms, including viruses, mycoplasmas, bacteria, fungi and oomycetes (Raaijmakers et al., 2010). (This specific topics will be further discussed in sub-section 6.6)

6.5.2. Motility

Movement of bacteria on surfaces has been extensively studied *in vitro* and several distinct forms have been recognized, which include swimming, swarming and twitching (Harshey, 2003; Henrichsen, 1972). By changing the viscosity of surfaces, biosurfactants can influence cell differentiation and motility. Nevertheless, no comprehensive studies concerning the influence of lipopeptides produced by *Bacillus* spp. in motility have been performed (Allison et al., 1993; McCarter & Silverman, 1990).

6.5.3. Plant pathogenesis and induction of systemic resistance

Recent studies have also shown that some lipopeptides have beneficial effects on plants, throughout the stimulation of the plant immune system. Indeed, purified fengycins and surfactins, but not iturins, induced significant protection in bean and tomato leaves against the fungal pathogen *Botrytis cinerea* (Ongena et al., 2007). Moreover, Wang et al., and Kim et al., (Kim et al., 2007; Wang et al., 2007) showed that some lipopeptides from nonpathogenic *Bacillus* species can be perceived by plants to trigger a defense response. Nevertheless, to further exploit these potential beneficial effects, knowledge about the structural features and constituents of lipopeptides involved is required.

6.5.4. Biofilm formation and development

The attachment of single bacterial cells to surfaces and, after cell division and proliferation, the formation of dense aggregates by the secretion of polymers as polysaccharides and proteins results in a structure known as biofilm (Stewart & Franklin, 2008). The extracellular matrix of the biofilm protects bacteria against adverse environmental conditions (Hall-Stoodley et al., 2004), and biofilms can also provide protection against protozoa predation and are a niche for horizontal gene transfer (Danhorn & Fuqua, 2007).

In the case of *Bacillus* spp., LPBSs play an important role in surface attachment and biofilm formation, although the effect may vary according to the type of lipopeptide, which could be partially attributed to differences in their physicochemical properties and to the potential effects of these compounds on hydrophobicity of the cell surface and/or in the substratum. Hofemeister et al. (Hofemeister et al., 2004) showed that surfactin was required for biofilm formation of *B. subtilis* strain A1/3, where the production of the lipopeptide appears important but not always sufficient for pellicle formation, as observed in a comparative study of five different *B. subtilis* strains (Chollet-Imbert et al., 2009). A crucial role for surfactin has also been demonstrated in the formation of aerial fruiting bodies that represent another type of structured bacterial communities (Branda et al., 2001).

Taking into account the great structural diversity of LPBSs described, differences in hydrophobicity, cell surface charge, substratum, ionic conditions of pH the role of

particular molecules in biofilm formation may be distinct (de Bruijn et al., 2008; Neu, 1996).

Surfactin can also act as a signalling molecule in triggering cannibalism and matrix formation in biofilms (López et al., 2009a; López et al., 2009b). Thus, biofilm formation was stimulated by surfactin and also by other molecules that cause potassium leakage, whereas other compounds as iturin failed to induce multicellularity (López et al., 2009a). Moreover, Lopez et al. (López et al., 2009b) showed that the cannibal/matrix-producing can also be created in response to antimicrobial compounds produced by other microorganisms. Based on these findings, they hypothesized that other organisms, which produce compounds that mimic the action of surfactin, may activate toxin production and biofilm formation in *B. subtilis* as a defense mechanism (López et al., 2009b).

These compounds may also adversely influence the attachment to surfaces and biofilm formation by other microorganisms, suggesting that lipopeptides may unfavorably affect the early stages of biofilm formation and, in some cases, can suppress existing biofilms of other microorganisms (Fracchia et al., 2012).

6.5.5. Chelation of metal ions and degradation of xenobiotics

Chelation of metal ions has been described for several biosurfactants, including lipopeptides and rhamnolipids. Actually, the capacity for lipopeptides chelation can be affected by structural changes. For example, for surfactin, it was shown that when the leucine at position 2 was substituted by isoleucine, a threefold increase in affinity for Ca^{2+} occurred, possibly by an increase in accessibility of the acidic side chains and carboxylate groups that organize the calcium-binding site (Grangemard et al., 1997). Additionally, binding of surfactin to Ca^{2+} resulted in a conformational change of the peptide moiety allowing its incorporation into a phospholipid bilayer (Maget-Dana & Ptak, 1995). The actual functions of LPBSs in relation to metal chelation and degradation of xenobiotics are not clear, and even the benefits to the producing bacteria need to be clarified. Possible hypothesis are: i) a protective role due to chelation of metal ions; ii) bacteria use the biosurfactants for sequestering specific metal ions as micronutrients. With respect to the degradation of xenobiotics, lipopeptides and other biosurfactants may provide access to persistent aromatic compounds with low solubility in water and use these compounds as carbon and/or nitrogen sources (Ron & Rosenberg, 2002).

6.6. Applications of LPBSs

6.6.1. Lipopeptides in pharmaceutical industry

The rapid increase and incidence of bacterial infections, particularly of multidrug resistant (MDR) bacteria, renewed the interest in the development of novel antibiotics for infection control purposes (Mandal et al., 2013). Nature and its huge biodiversity seems to be an endless source of compounds containing unique chemical structures, some of them having been exploited for this purpose. However, their commercial exploitation depends largely on a cost-effective production and demonstration of low toxicity. The most well-studied biosurfactant (surfactin) has been highlighted for different applications (Figure 19).

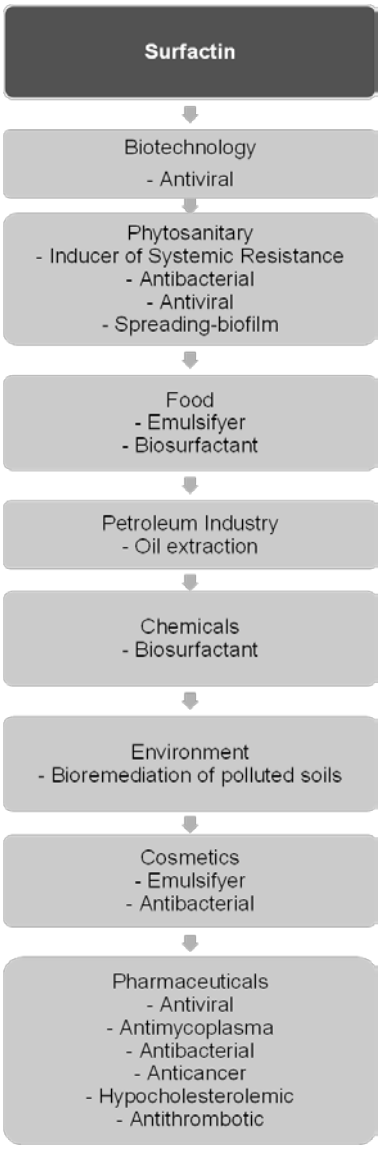


Figure 19. Application sector and exploited properties of surfactin.

A high number of scientific publications and patents have been developed but its application as an antibacterial agent is slowered by high production costs and also the suspected toxicity (From et al., 2007). Thus, this section will focus on the application of biosurfactant agents produced by *Bacillus* spp. in a pharmaceutical overall perspective.

Lipopeptides present potent antibacterial activity due to the ability of these molecules to self-associate and form a pore-bearing channel or micellar aggregate inside the lipid membrane (Deleu et al., 2008). Previously identified lipopeptides and their presumptive activity for microbial infection control are summarized in Table 8 and Table 9.

Surfactin, in particular, acts non-specifically causing membrane disruptions on both Gram-positive and Gram-negative bacteria (Lu & Mosier, 2007), which has recently been suggested as the next generation of antibiotics (Rodrigues & Teixeira, 2010).
Similar bioactive fractions from the marine *B. circulans*, present antimicrobial activity against various Gram-positive and Gram negative pathogens, including *Micrococcus flavus, B. pumilis, Mycobacterium smegmatis, Acinetobacter calcoaceticus, Bordetella bronchiseptica* and several *Enterobacteriaceae* species (Das et al., 2008).

In addition to surfactin, *B. subtilis* strains produce other bioactive peptides (fengycin and iturin families) with great potential of antimicrobial activity, namely iturin A (Besson et al., 1978; Peypoux et al., 1978; Peypoux et al., 1984), mycosubtilin (Peypoux et al., 1986) and bacillomycin (Peypoux et al., 1984), all of which membrane-active agents.
Lichenysin, pumilacidin and polymyxin B (Landman et al., 2008; Grangemard et al., 2001; Yakimov et al., 1995; Naruse et al., 1990;) are other examples of antimicrobial lipopeptides produced by *B. licheniformis*, *B. pumilus* and *B. polymyxa*, respectively.
Polymyxin B, in particular, due to its high affinity to the lipid moieties of lipopolysaccharide, presented antibacterial activities against a wide range of Gram-negative pathogens (Landman et al., 2008). Being a cationic agent, it binds to the anionic bacterial outer membrane, leading to a detergent effect that disrupts membrane integrity.

Another promising example of an antimicrobial lipopeptide that is under commercial use is daptomycin (Cubicin®), with applications in the treatment of skin infections (Seydlová & Svobodová, 2008). Moreover, Daptomycin, produced by *Streptomyces roseosporus* has a potent activity against clinically-relevant methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), glycopeptide-intermediate-susceptible *S. aureus* (GISA), coagulase-negative *Staphylococci* (CNS), and penicillin-resistant *Streptococcus pneumoniae* (PRSP) pathogens (Tally et al., 1999). In addition,

glycolipids, both rhamnolipids (Benincasa et al., 2004) and sophorolipids (Van Bogaert et al., 2007) have also shown remarkable antimicrobial activities particularly against *B. subtilis* (Benincasa et al., 2004), *B. cereus*, *S. aureus*, *Micrococcus luteus*, *Mucor miehei* and *Neurospora crassa* (Nitschke et al., 2010) and also MRSA (Mimee et al., 2009).

Studies also showed that surfactin has an anti-mycoplasma activity allowing the specific inactivation of mycoplasmas contaminating mammalian cells, without significantly damaging effects on cell metabolism (Fassi et al., 2007; Kumar et al., 2007; Vollenbroich et al., 1997). It has also been shown that this compound exhibit a synergistic effect in combination with enrofloxacin, which resulted in mycoplasma-killing activity of about two orders of magnitude greater when compared with the molecules used separately (Fassi et al., 2007).

Caspofungin (Arendrup et al., 2012; Letscher-Bru & Herbrecht, 2003), pneumocandin (Schwartz et al., 1988), aculeacin (Mizuno et al., 1977) and other related compounds have been associated with antifungal activities (Hino et al., 2001). Other lipopeptides presenting this function include ciclic lipopetides (surfactin, iturin and fengycin) (Chen et al., 2009; Kim et al., 2010; Snook et al., 2009) and glycopeptides (cellobiose lipids and rhamnolipids) (Banat et al., 2010).

Surfactin and its analogues also present a strong antiviral activity (Naruse et al., 1990), especially against enveloped viruses (retroviruses and herpes viruses), suggesting that this activity may be due to the formation of ion channels on the virus envelope, and consequently the lost of viral proteins (Seydlová & Svobodová, 2008; Jung et al., 2000). Additionally, *in vitro* experiments showed that surfactin and fengycin produced by a *B. subtilis* strain were able to inactivate cell-free virus of porcine parvovirus, pseudorabies, newcastle disease and bursal disease and, which could effectively inhibit its infection and replication (Huang et al., 2006).

In addition, few peptides are already marketed as vaccines. In fact, most of them are applied in HIV immunization, combining an HIV derived peptide and a lipid tail (Rizos et al., 2007; Durier et al., 2006). Moreover, Monash Institute of Pharmaceutical Sciences has recently developed a novel lipopeptide antibiotic to target Gram-negative multi-drug-resistant bacteria, promising to be less apoptopic than polymyxins and with a longer half-life (patent no. PCT/AU2010/000568).

Different studies have also demonstrated that particular lipopeptides (mainly surfactins) have anti-inflamatory, anti-tumoral or immuno-modulatory properties (Cao et al., 2010; Park et al., 2010; Park & Kim, 2009;). The anti-inflamatory activity has been associated with oxid nitric induction and reduction of pro-inflammatory cytokines, including tumor necrosis factor-α and interleukin (IL)-1β, IL-6 and IL-12 (Byeon et al., 2008; Park et al., 2010). The anti-tumoral activity is related with the involvement of lipopeptides in signal transduction, cell differentiation and cell immune responses, having been demonstrated that surfactin induces apoptosis in human breast cancer (Cao et al., 2011; Osada, 1998). Moreover, Park et al. (2009) also demonstrated a potent immunosuppressive capability for surfactin, which suggests an important therapeutic implication to transplantation and also to autoimmune diseases, including allergy, arthritis and diabetes (Park & Kim, 2009).

The colonization of medical or prostheses with microbial biofilms is a hazardous occurrence, particularly if the bacteria become highly resistant to antibiotic, since they can seriously compromise antimicrobial therapy (Morikawa, 2006). Several reports have suggested that, in addition to their direct action against pathogens, biosurfactants are able to interfere with biofilm formation, modulating microbial interaction with interfaces (Rodrigues et al., 2007; Rasmussen & Givskov, 2006; Rodrigues et al., 2006b; Rodrigues et al., 2006c; Merk et al., 2005; Federle & Bassler, 2003; Neu, 1996;), which seems to be an attractive alternative to more conservative approaches (coating of medical surfaces with antimicrobial agents) (Basak et al., 2009; von Eiff et al., 2005). Indeed, biosurfactants have the advantages of precisely target biofilm growth, while causing no adverse toxicity in the environment.

Surfactin, for example, has shown to be an important biofilm controlling agent, able to inhibit biofilm formation of *Salmonella* Typhimurium*, Salmonella enterica*, *E. coli* and *Proteus mirabilis* in polyvinyl chloride wells, as well as vinyl urethral catheters (Mireles et al., 2001).

A recent study showed that two lipopeptide biosurfactants produced by *B. subtilis* V9T14 and *B. licheniformis* V19T21, evidenced the capacity to selectively inhibit biofilm formation of *S. aureus* ATCC 29213 and *E. coli* CFT073 on polystyrene surfaces (Rivardo et al., 2009). Chemical characterization of V9T14 lipopeptide biosurfactant carried out by Liquid Chromatography – Electrospray Tandem Mass Spectrometry (LC/ESI-MS/MS) revealed that it was composed of 77% of surfactin and of 23% of fengycin (Pecci et al., 2010). In another study, the V9T14 biosurfactant in association with some antibiotics led to a synergistic increase in the efficacy of antibiotics against *E. coli* CFT073 biofilm inhibition and, in some combinations, to the total eradication of the uropathogenic strain biofilm producer (Rivardo et al., 2011).

Moreover, bioproducts recovered from marine *B. pumilus* and *B. indicus* significantly inhibited the initial attachment process and biofilm formation of mature biofilms of *Vibrio* spp. strains (Nithya & Pandian, 2010).
Thus, anti-adhesive activity of biosurfactants against several pathogens highlights their potential applicability as coating agents for medical insertional materials, which can contribute to a reduction in a large number of nosocomial infections avoiding the use of synthetic drugs and/or chemicals.

Lipopetides can also be used in the context of drug delivery due to their properties of detergency, emulsification and foaming (Faivre & Rosilio, 2010). Recently, fengycin and surfactin were successfully used in the increasing of aciclovir concentration in the epidermis (Nicoli et al., 2010). Moreover, microemulsions of water/oil/surfactant are being explored as liquid vehicles for future drug delivery systems due to its long-term stability, easy preparation and high solubilization capacity (Date & Nagarsenker, 2008).

Gene transfection into the cells is a fundamental technology not only for molecular and cellular biology processes but also for clinical gene therapy (Mulligan et al., 2014). Although several methods for gene transfection have been investigated (Mulligan et al., 2014), More efficient and safe systems are desirable for gene transfection (Ueno et al., 2007), and lipofection (based on the use of liposomes) is considered a promising method for introduction of a foreign gene into the targeted cells (Nakanishi, 2003; Inoh et al., 2001). In fact, glycolipids have received particular attention in this area, when compared with lipopepides produced by *Bacillus* spp. (Ueno et al., 2007; Igarashi et al., 2006; Inoh et al., 2001) reported that MEL-A promoted DNA transfection efficiently by induction of membrane fusion between the target cells and the cationic liposomes.

In addition to the known antimicrobial agents comprising low molecular weight antimicrobial substances and bacteriocins, probiotics have long been known for their ability to interfere with the adhesion and formation of pathogen biofilms to epithelial cells of urogenital and intestinal tracts (Reid et al., 2001) by releasing of surface active molecules (Gudiña et al., 2010; Rodrigues et al., 2006b; Rodrigues et al., 2006c). Several studies pointed out that probiotic microorganisms (mainly *Streptococcus thermophilus* and *Lactobacillus* spp. strains) and their biosurfactants may antagonize the growth and the development of potentially pathogenic microorganisms including *S. aureus*, *S. epidermidis*, *Streptococcus* spp., *E. faecalis, Candida albicans, Candida tropicalis* (van Hoogmoed et al., 2000; Busscher et al., 1997). Thus, considering their importance for human health and their recognized safety, probiotic organisms may represent a safe and effective intervention for infection control purposes.

Probiotics themselves or their products (biosurfactants), could be applied to patient care equipment, such as tubes or catheters, with the aim of decreasing the colonization of these sites by nosocomial pathogens and potentially impede a central step in the pathogenesis of nosocomial infections (Falagas & Makris, 2009).

Table 8. List of identified active lipopeptides produced by *Bacillus,* which can be used for infection control purposes.

Lipopeptides	Source	Activity	Reference
Surfactin	*B. subtilis*	*Enterococcus faecalis* ATCC 2912 *Lactococcus garviae* KCCM 40698 *Streptococcus parauberis* DSM 6631 *Flexibacter tractuosus* ATCC 23168 *Vibrio harveyi* ATCC 14126	Kim et al., 2009
Mycosubtilin		*Micrococcus luteus*	Peypoux et al., 1979
Fusaricidin A		MRSA *S. aureus* *S. epidermidis* Vancomycin-resistant *E. faecium*	Stawikowski & Cudic, 2009
Lichenycin		*Corynebacterium variabiliss* *Acinetobacterr* sp.	Nerurkar, 2010
Bacillomycin D		Antifungal (*Sclerotinia sclerotiorum*)	Kumar et al., 2012
Iturin A		Antifungal (*Fusatium oxysporum*)	Thimon et al., 1995 Yuan et al., 2011

Table 9. Recent patents on lipopeptide as infection control agents.

Patent no.	Year	Patent Name	Source	Inventors
US 2011/0224129 A1	2011	Lipopeptide compounds and their use	Modified friulimicin	Boyce et al.
US 2011/0030103 A1	2011	Lipopeptides and lipopeptide synthetases	Engineered lipopeptide synthetase polypeptide	Reznik et al.
US 7868135 B2	2011	Composition of lipopeptide antibiotic derivatives and methods of use thereof	Amphomycin or aspartocin derivative	Cameron et al.
US 7795207 B2	2010	Lipopeptide compositions	Cyclodextrin derivatives	Labischinski et al.
US 2010/0184649 A1	2010	Novel antibacterial agents for the treatment of Gram-positive infections	Isolated combined compounds	MetCalf,III et al.
US 7671011 B2	2010	Antimicrobial and anticancer lipopeptides	Conjugate lipopeptide	Shai et al.
US 7655623 B2	2010	Dab^9 derivatives of lipopeptide antibiotics and methods of making and using the same	Synthetic amphomycin-type	Fardis et al.
US 2009/0233870 A1	2009	Antimicrobial peptides	Synthetic	Blondelle et al.
US 2009/0202519 A1	2009	Compositions and methods for treating Gram positive bacterial infection in a mammalian subject	Synthetic	Beutler et al.
US 7408025 B2	2008	Lipopeptides as antibacterial agents	Synthetic	Hill et al.
US 6911525 B2	2005	Lipopeptides as antibacterial agents	Synthetic	Hill et al.
US 6750199 B2	2004	Antimicrobial sulfonamide derivatives of lipopeptide antibiotics	Lipopeptide derivatives	Curran et al.
US 6696412 B1	2004	High purity lipopeptides, lipopeptides micelles and processes for preparing same	Daptomycin	Kelleher et al.
US 6624143 B1	2003	Calcium salts of lipopeptide antibiotics, method for producing same and their use	Synthetic	Vértesy et al.
US 6511962 B1	2003	Derivatives of laspartomycin and preparation and use thereof	Laspartomycin	Borders et al.

6.6.2. Lipopeptides in cosmetic industry

In the last few decades, several lipopeptides have been extensively used in the cosmetics industry due to their exceptional surface properties, presenting anti-wrinkle and moisturizing activities on human skin (Kanlayavattanakul & Lourith, 2010). Moreover, they are also characterized by their capacity of stimulate the producing new collagen and elastin and their antioxidant properties (Singh & Cameotra, 2004b) delaying lipid peroxidation, in addition to the healthy skin physiology support, with potential application in types of facial cosmetics and lotions. Nevertheless, few cosmetic companies have already developed and marketed their lipopeptide-formulated products that directly help to stimulate collagen and elastin production as anti-aging agents (Kanlayavattanakul & Lourith, 2010) due to their high production cost.

Surfactin has received greatest attention due to its low CMC value and consequent suitability for topical dermatological application (Kanlayavattanakul and Lourith, 2010). In fact, these compounds have attested safe application and minor irritation to human skin being used in dermatological products and in cleansing cosmetics (Kanlayavattanakul & Lourith, 2010).

To expand biotechnology to a profitable industry, in the future, genetic manipulation of organisms should be essential for high yield and high quality production of lipopeptides.

6.6.3. Lipopeptides in food industry

In the food industry, lipopeptides are mainly used as emulsifiers in the processing of raw materials and as preservatives during processing step to avoid microbial pathogenic spoilage. Among bio-preservatives, several antimicrobial compounds (more than 500) have been accepted to effectively control food pathogens (Stein, 2005; Ricca et al., 2004). Nevertheless, the use of these antimicrobial compounds as food preservatives, proteins and/or peptides, are limited due to their sensitivity to proteases. Iturins, fengycins and surfactins families are known as protease resistant, exhibiting strong growth inhibition of a wide range of pathogens (Touré et al., 2004). Notwithstanding, in the baking industry, surfactins and rhamnolipids are also used to maintain stability, texture, and volume, and also to help in the emulsification of fat tissue in order to control fat globule agglomeration (Campos et al., 2013).

Additionally, biosurfactants can have also benefit on its use as pre-treatment of material surfaces found in food-processing environments. In fact, pathogenic bacteria implicated in food-borne illness outbreaks are able to form biofilms on food contact surfaces that are more resistant to sanitation than free-living cells (Kim et al., 2006; Stepanovic et al., 2004; Kalmokoff et al., 2001). Thus, pre-conditioning surfaces using microbial surface-active compounds may be an interesting strategy for preventing the adhesion of food-borne pathogens to solid surfaces. Recently, Nitschke et al., (Nitschke et al., 2009) investigated the effect of rhamnolipid and surfactin biosurfactants on the adhesion of the food pathogens *Chronobacter*, *Listeria monocytogenes* and *S. enteritidis* to polypropylene and stainless steel surfaces (Nitschke et al., 2009) and concluded that preconditioning with surfactin, rather than rhamnolipid, caused a reduction in the number of adhering cells particularly of *L. monocytogenes* and *Chronobacter* on stainless steel. Thus, lipopeptides satisfy several characteristics of beneficial additives for emulsifying, antiadhesive, and antimicrobial activities which suggest their application as multipurpose ingredients, additives or even in developing strategies to prevent or delay microbiological colonization of industrial plant surfaces used in foodstuffs preparation.

6.6.4. Lipopeptides in biotech and nanotech industry

In a market where biosurfactants production is highly expensive, efforts have been conducted with special emphasis on the utilization of various cheap agro-industrial substrates and large-scale production (Mulligan et al., 2014; Winterburn & Martin, 2012) for their management of cost-effective production. In this context, some efforts have been made to genetically manipulate or alter the lipopeptide structure to selectively enhance antimicrobial activities (Koglin et al., 2010) and reduce their production cost. Lipopeptides can also be used in the synthesis of metal-bound nanoparticles as an alternative environmentally friendly technology (Sharma et al., 2009; Ariga et al., 2007; Shimizu et al., 2005) due to their ability to self-assemble into hierarchically ordered structures. Reddy et al. (Reddy et al., 2009) successfully synthesized surfactin-mediated gold and silver nanoparticles using *B. subtilis.* According to the authors, nanoparticles were stabilized by the surface-active molecules i.e., surfactin or other biomolecules released into the solution by *B. subtilis*.

Surfactin produced by *B. amyloliquefaciens* KSU-109 was also used for the synthesis of cadmium sulfide nanoparticles which remained stable up to six months without compromising their functionality (Singh et al., 2011). This kind of molecules works as

semiconductors with unique optical properties and unable photo-luminescence, allowing potential applications in solar energy conversion, nonlinear optical, photoelectrochemical cells and heterogeneous photocatalysis (Singh et al., 2011). Such simple, inexpensive and environmental friendly procedure of obtaining surfactin offers a further advantage of use in nano-biotechnology for the large-scale production of highly stable metal nanoparticles.

Moreover, some potential therapeutic applications of these compounds have been exploited and include anti-HIV, anti-angiogenesis, antimalarial, anti-arthritic, anti-fungal, anti-inflammatory, anti-viral, anti-angiogenesis and anti-platelet activities (Mulligan et al., 2014).

6.6.5. Environmental application

Organic and/or inorganic chemical contaminants are highly stable in soil , preventing its remediation and influencing negatively the living organisms (Mulligan et al., 2001b) (Mulligan & Yong, 2004). The use of biosurfactants can improve bioremediation effectiveness of hydrocarbon contaminated environments, by the increase of the hydrocarbon bioavailability for microorganisms, and/or by its interaction with the bacterial cell surface, allowing the association of hydrophobic substrates with bacterial cells (Mulligan & Gibbs, 2004). On the other hand, heavy metal contaminated soil bioremediation by biosurfactant activity seems to be associated with the ability to form complexes with metals and with chelation properties (Aşçı et al., 2008; Juwarkar et al., 2007; Mulligan & Gibbs, 2004; Mulligan et al., 2001b; Singh & Cameotra, 2004a).

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CHAPTER 2

Diversity, differentiation by high-throughput methods and biosafety of *B. pumilus* group species

2.1. Phylogenetic and clonality analysis of *Bacillus pumilus* group members

Publications:

Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a highly heterogeneous population of different closely related species and clones

In this study, throughout phylogenetic analysis of a comprehensive collection of *Bacillus pumilus* isolates we demonstrate that most of them are misnamed, belonging to *Bacillus safensis* or to *Bacillus altitudinis.* The results obtained also uncovered habitats and a human health or biotechnological relevance of *B. safensis* and *B. altitudinis* previously reported to *Bacillus pumilus,* e.g. human and animal probiotic and plant growth promoter. Moreover, for the first time we demonstrated the genomic plasticity of these species, which can justify their widespread and adaptation capacity to different niches.

Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a highly heterogeneous population of different closely related species and clones

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Running title: Species and clonal diversity within *Bacillus pumilus*

Keywords: *Bacillus pumilus*, *Bacillus safensis*, *Bacillus altitudinis*, diversity, 16S rRNA gene, *gyrB* gene, *rpoB* gene, PFGE; phylogeny

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Abstract

Bacillus pumilus is a Gram-positive bacterium with a wide range of attributed applications, namely as a plant growth promoter, animal and human probiotic. Although rarely, a putative role in human diseases, namely in food poisoning or as anthrax-like cutaneous infectious agent, has also been reported. This species is difficult to distinguish from its closely related species on the basis of phenotypic or biochemical characteristics and 16S rRNA gene sequences. In this study, by phylogenetic analysis of *gyrB* and *rpoB* gene sequences of a collection of previously identified *B. pumilus* isolates, we demonstrated that most (93%, 38 of 41 isolates) of them belong to *B. safensis* or to *B. altitudinis*. Moreover, we extend the recognized habitats and unveil a human health or biotechnological relevance (e.g. as PGPR or implicated in food poisoning) of *B. safensis* and *B. altitudinis*. Additionally, we demonstrated that these species encompasses a clonally diverse population, which can justify their great adaptation ability to different niches, with evidence of a particular clonal host specificity.

Introduction

Currently, the polyphyletic *Bacillus* genus is grouped in six taxa based on phenotypic characteristics and on 16S rRNA gene (Stackebrandt *et al.,* 2008). Indeed, it has been subjected to frequent restructuration's with the continuous recognition of new species (Shivaji *et al.,* 2006; You *et al.,* 2013; Liu *et al.,* 2013; Lai *et al.,* 2014). One of the groups that has been suffering constant taxonomic adjustments is RNA group I, to which belong *Bacillus pumilus* group species (Earl *et al.,* 2008; Jeyaram *et al.,* 2011). Members of this last group encompass *B. pumilus*, the first species described, *Bacillus safensis, Bacillus altitudinis, Bacillus stratosphericus, Bacillus aerophilus* and *Bacillus xiamenensis* (Satomi *et al.,* 2006; Liu *et al.,* 2013; Lai *et al.,* 2014).

The ubiquity of *B. pumilus* group species range from diverse terrestrial and marine settings (Satomi *et al*., 2006; Freitas *et al*., 2008; Liu *et al*., 2013; Lai *et al*., 2014), and air of high altitudes (Shivaji *et al.,* 2006), with spores production capacity pointed out as the crucial condition to facilitate their adaptation into different ecological niches (Stackebrandt *et al.,* 2008).

A wide range of applications has been attributed to *B. pumilus*, including animal and human probiotics (Sanders *et al.,* 2003; Hong *et al.,* 2005; EFSA 2011), phytosanitary products (Pérez-García *et al.,* 2011) or plant growth promoters (Joo *et al.,* 2004). Although rarely, *B. pumilus* has been also associated with food poisonings and human infections including anthrax-like cutaneous lesions (Castagnola *et al.,* 2001; Haymore *et al.,* 2006; Ozkocaman *et al.,* 2006; Bentur *et al.,* 2007; From *et al.,* 2007; Tena *et al.,* 2007; Johnson *et al.,* 2008). Contrastingly, there is scarce data regarding the relevance of remaining *B. pumilus* group species (Agbobatinkpo *et al.,* 2013, Ahaotu *et al.,* 2013).

Despite significance of the *B. pumilus*, there are no comprehensive studies on the clonal structure of this species, with a recent assay using MLSA (Multilocus Sequence Analysis) not demonstrating a suitable intra-species resolution power (Liu *et al.,* 2013).

Assessing the clonal diversity of previously identified *B. pumilus* isolates, we realized the difficulty in distinguishing this member at the species level, on the basis of phenotypic, biochemical characteristics and 16S rRNA genes sequence. Sequencing of β-subunit of DNA gyrase (*gyrB*) and β-subunit of RNA polymerase (*rpoB*) genes, have proven to be

useful for taxonomic resolution of closely related species and were previously suggested for classification of *Bacillus* spp. members (Konstantinidis *et al.,* 2006; Adékambi *et al.,* 2008). Recently, these molecular biomarkers were assessed to position phylogenetically *B. pumilus* group species (Liu *et al.,* 2013).

In this study, a collection of terrestrial isolates previously identified as *B. pumilus* was analyzed phylogenetically, based on *gyrB* and *rpoB* genes. Intra-species diversity of the depicted species was also assessed to unveil their genomic plasticity and clonal dispersion in different settings.

Material and Methods

Isolates collection and identification

A comprehensive collection of forty-one *B. pumilus* isolates from different origins previously identified by phenotypic and biochemical characteristics and 16S rRNA gene sequence were studied. It comprises isolates recovered from different terrestrial sources and geographic locations, including food samples (n=6) (Norway, Italy and Africa), plant growth-promoters (PGPR) (n=4) (USA), gastropods normal flora (n=3) (Portugal), and medicines (n=11) and cosmetic (n=4) contaminated products (Portugal). Additionally, 11 *B. safensis,* including *B. safensis* FO-36b^{T}, kindly provided by NASA collection and recovered from clean room environments from Mars Odyssey (USA), and *B. pumilus* ATCC 7061^{T} and 14884 were included for phylogenetic comparisons (Table 1).

Sequencing of 16S rRNA, β-subunit of DNA gyrase (*gyrB*) and β-subunit of RNA polymerase (*rpoB*) genes

The DNA was prepared using InstaGene^{TM} Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and was used directly for PCR amplification. Gene fragments were amplified according to primers pairs and PCR conditions outlined in Table 2. For each isolate, nucleotide sequences of the 16S rRNA, *gyrB* and *rpoB* genes were obtained by sequencing of amplified products. All sequences were deposited in the GenBank database; the accession numbers are listed in Table 1.

Phylogenetic analysis

The sequences of 16S rRNA, *gyrB* and *rpoB* genes obtained in this study were compared with the ones available in GenBank nucleotide and protein databases using BLASTN and BLASTX algorithms. Nucleotide sequences were aligned and species similarity score for each gene were generated using MEGA version 5.2.2 (http://www.megasoftware.net/) (Tamura *et al.,* 2011). Phylogenetic trees were constructed from the alignment of similarity scores using neighbour-joining (NJ) (Saitou *et al.,* 1987) method. During the analysis of alignments, genetic distances were estimated using Kimura´s 2-parameter

model (Kimura, 1980), where all substitutions were included in pairwise distance calculations. The reliability of internal branches was assessed from bootstrap based on 1000 resamplings. In order to establish phylogenetic relationships, homologous nucleotide sequence data from *B. pumilus* group members were also included. Sequences (n=72) found deposited on GenBank (accession numbers detailed in table 1 and 3) correspond to *B. pumilus* strain SAFR-032, *B. altitudinis* 41KF2b^T. and *B. pumilus* (n=11)*, B. safensis* (n=11) and *B. altitudinis* (n=48) recovered from marine environments. Unfortunately, sequences of *B. stratosphericus*, *B. aerophilus* and *B. xiamenensis* are not available in the databases neither the isolates available from public collections, therefore it was not possible their inclusion for phylogenetic purposes. Moreover, *B. subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* DSM7^T*, B. licheniformis* ATCC 14580^T and *B. cereus* ATCC 14579^T were used as outgroups. Therefore the total number of strains analyzed was one hundred and seventeen.

Genetic diversity analysis

Intra-species diversity was assessed by a modified PFGE protocol (Kaufmann, 1998), using a higher bacterial cell concentration and *Apa*I (New England Biolabs, Beverly, Mass.) for DNA macrorestriction. Briefly, *Bacillus* isolates were grown overnight in TSB (Tryptic Soy Broth, BD, Germany) in a shaking water bath at 30ºC and collected by centrifugation during 12 min at 2200 g^{-1}. Cells were washed with 0.5 mL of PET-IV buffer [10 mMTris-HCl (Sigma-Aldrich, St. Louis, MO) and 1M NaCl (Sigma-Aldrich, St. Louis, MO), pH=7.5], resulting in a high cell concentration, and subsequently mixed with an equal volume of molten (55ºC) 1.6 % low-melting point agarose (SeaKem Gold, SKG) (FMC BioProducts, Denmark). Plug sections were included in a 1.6 % agarose with running conditions of 3.5s – 15s for 27h at 14ºC in a Tris Boric Acid-EDTA (TBE, 0.5X) supplemented with thiourea (100µM; Sigma-Aldrich, St. Louis, MO).

*Apa*I profiles were analyzed by InfoQuest FP version 5.4 software (Bio- Rad Laboratories, USA), and the percentage similarity was calculated by applying the unweighted-pair group method using average linkages (UPGMA) algorithm based on the Dice coefficient, and 1.0% of band tolerance and 1.0% of optimization. PFGE-types were defined using Tenover criteria (Tenover *et al.*, 1995).

Results and Discussion

Phylogenetic analyses

Phylogenetic tree analysis based on 16S rDNA gene sequences (≅ 1,500bp) showed that the isolates tested in this study clustered as well as other *B. pumilus* group members available in GenBank, in a separated branch from the remaining *B. subtilis* group species (*B. subtilis* subsp. *subtilis*, B*. amyloliquefaciens* and *B. licheniformis*), and also from *B. cereus*, with an overall 93.4% of homology achieved among all isolates (Fig.1). Moreover based on genetic distances calculated to 16S rRNA gene for all *B. pumilus* group members tested, phylogenetic tree revealed a level of homology ranging between >99.4-100% (Fig.1 and Fig. 4a).

Interestingly, and despite the high homology, 16S rRNA topology tree shows that most of isolates from the terrestrial collection characterized in this study, did not organize within the groups enclosing mostly of *B. altitudinis*, *B. pumilus* or *B. safensis* deposited in GenBank (Fig.1). It's worth to mention that these groups include mostly 16S rRNA gene sequences from isolates recovered in the same location, from which we lack information on their clonal relationships. Contrastingly, a clonal diversity was observed through the use of PFGE, which will be further discussed in the genetic diversity analysis section.

Due to limitations of 16S rRNA gene sequences to decipher relationships at the species level, as previously recognized for strains belonging to *B. subtilis* group (Satomi *et al.,* 2006; Wang *et al.,* 2007), *gyrB* and *rpoB* genetic markers, which presented a higher variability, have been used (Satomi *et al.*, 2006; Wang *et al.,* 2007; Liu *et al.,* 2013).

Phylogenetic affiliation based on *gyrB* gene sequences (≅1070 bp in length) was shown in Figure 2. Accordingly to the bacterial genus under study, different sequence similarity values have been required for taxonomic assignments on the basis of the *gyrB* gene (La Duc *et al.,* 2004). In the case of *B. subtilis* complex, where *B. pumilus* and consequently, *B. safensis* and *B. altitudinis* belong, around 95% similarity of *gyrB* gene was accordant with 70% of DNA-DNA relatedness (Wang *et al.,* 2007). A similar cut-off, 95-96% of homology, to distinguish at species level the *B. pumilus* group members was recently proposed (Liu *et al.,* 2013).

Analysis disclosed from Figure 2, delineated seven distinct phylogenetic clusters among all sequences analyzed, including *B. subtilis subsp. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. cereus, B. pumilus, B. safensis, B. altitudinis*, with an overall 72.5% of homology. Moreover, *gyrB* sequence-based phylogenetic topology clearly divided the *B. safensis, B. pumilus*, and *B. altitudinis* into three distinct clusters, $gyrB_1$, $gyrB_2$ and $gyrB_3$, respectively.

The group $gyrB_1$, which included *B. safensis* FO-36b^T, also comprised 12 isolates previously assigned to *B. pumilus* recovered from food samples, plants, gastropods normal flora, and medicines and cosmetic products, and 26 isolates from marine settings, with 98% of homology supported by a bootstrap value of 100%. This group is more closely related to *B. pumilus* isolates grouped in $gyrB_2$ (genetic distance of 0.094), than to *B. altitudinis* ones ($gyrB_3$) (genetic distance of 0.104), which were supported by bootstrap values of 99% and 97%, respectively. With the reassignment as *B. safensis* of several *B. pumilus* isolates identified in gastropods or used as PGRP we extended the recognized habitats and significance of *B. safensis*.

.Group $gyrB_2$ comprised the *B. pumilus* ATCC 14884 and 7061^T, the unique *B. pumilus* (SAFR-032) until now completely sequenced, 3 of 41 *B. pumilus* isolates characterized in this study recovered from medicine's and 14 marine isolates. The *gyrB* sequences of this clade shared 98.6% of homology. Moreover, this clade presented a genetic distance of 0.070 with group $gyrB_3$ and was supported by a bootstrap value of 100%. Therefore, these results demonstrated that *B. pumilus* are not the most widespread bacterial group among our collection, in contrast to general conception on this subject (Stackebrandt *et al.,* 2008).

Finally, $gyrB_3$, a third group, representing *B. altitudinis* included 9 isolates from the terrestrial collection tested, including a PGPR and a food poisoning isolate, and 49 from marine environments, which presented 98.8% of homology among them (bootstrap value support of 97%). Nevertheless, the topology of $gyrB_3$ tree seems to segregate our terrestrial isolates in a new branch as well as 4 isolates from marine sources (Ba28m, Ba52m, Ba56m and Ba73m). Results disclosed by its phenotypic and biochemical characterization support the distinction found between them and *B. altitudinis*. Further, genome sequencing and chemotaxonomic studies will elucidate their relatedness with *B. altitudinis*. Thus, this data extend the niches of *B. altitudinis* and unveils its relevance as a possible source of interesting bioproducts.

Analysis disclosed from *rpoB* gene analysis confirmed the previously clustering obtained by *gyrB* gene phylogenetic tree, presenting an overall congruent topology structure. According to Adékambi *et al.,* (2008), for complete *rpoB* gene, a cutoff of 97.7% and 98.2% could be applied to delineate the species and subspecies, respectively. In this study, two *rpoB* gene fragments were used for the phylogenetic assignments constructed using Neighbor Joining algorithm and Kimura´s 2-parameter model to calculate genetic distances between isolates: (i) the entire *rpoB* gene sequence and (ii) the hypervariable region, located between positions 2300 and 3300 pb. Our results showed that this last region of *rpoB* gene proved to be appropriated for the identification of these *B. pumilus* group species and the alignment pattern achieved was the same when compared with analysis of the entire gene. Thus, our comparison analysis was conducted using the hypervariable region of *rpoB* gene (Figure 3).

Global analysis of phylogenetic tree derived from the hypervariable region of this gene also revealed the presence of seven distinct clusters among all *Bacillus* isolates under study, which shared ≤97.7% of similarity between them, in accordance with the species cut-off defined. Furthermore, a marked differentiation was also observed among *B. pumilus* group members, grouping similarly the isolates into three main clades, herein $rpoB_1$, $rpoB_2$ and $rpoB_3$.

The group $rpoB_1$, comprise the same 38 *B. safensis* isolates as the correspondent *gyrB* sequence gene analysis, with a 99.2% of homology, supported by a bootstrap value of 97%. This group demonstrated a genetic distance of 0.025 with group $rpoB_2$ and 0.039 with group $rpoB_3$, which in accordance with *gyrB* gene sequence analysis and with the cutoff established to differentiate among species, clearly depicts three independent and closed species.

Also similar to *gyrB* gene topology, the $rpoB_2$ group, which included *B. pumilus*, was comprised of the same 17 isolates (herein with 99.1% of homology, supported by a bootstrap value of 99%). This group demonstrated a genetic distance of 0.034 with group $rpoB_3$.

Remarkably, group $rpoB_3$ seems to form the same segregation pattern achieved in *gyrB* gene analysis, related to those nine isolates recovered from terrestrial sources with some discrepancies in their neighbors (Fig. 3). These terrestrial isolates shared 98.7% of homology with *B. altitudinis* group, 96.6% with *B. pumilus* and 96.1% with *B. safensis*.

Congruently with the phylogenetic positioning revealed by *gyrB* gene, *rpoB* gene analysis reveals that just a few (3 of 41 isolates) of previously identified terrestrial *B. pumilus* isolates were correctly assigned, as mostly of them belong to *B. safensis* or to *B. altitudinis*.

Overall, genetic distances shown in Fig. 4b demonstrate that these two housekeeping genes presented remarkably higher resolution than the correspondent 16S rRNA gene to discriminate among *B. pumilus* group members. Moreover, and contrasting with the work of Liu *et al.* (2013), our analysis clearly revealed that hypervariable region of *rpoB* gene is an adequate marker to infer phylogenetic discrimination among closely related species of this group, probably due to the inclusion of a larger number of isolates of terrestrial origin, which strengthens the taxonomic affiliation.

Finally, trees constructed based on amino acid sequence of RpoB and GyrB (supporting information material, Figures S1 and S2) showed that most nucleotide substitutions were synonymous, i. e., did not result in amino acid changes, which is not surprising since *rpoB* and *gyrB* encoded proteins vital for cellular function (Case *et al*., 2007). Thus, *B. pumilus* group RpoB and Gyr*B* proteins analysis shared the same topology as the correspondent genes analysis.

Clonal diversity analysis

In order to assess the intra-species diversity within the *B. pumilus* group species assigned by *gyrB* and *rpoB* gene sequencing, macrorestriction followed by pulsed-field gel electrophoresis (PFGE) was performed. The discriminatory power of PFGE in the population analysis of different bacterial species is recognized and it was not superseded by MLSA in short time period bacterial evolutionary studies (Wolska 2012; Freitas *et al.*, 2013). Moreover, and despite the interest of MLSA, especially for long-term investigations of bacterial population structures, an attempt to establish a scheme for *B. pumilus* group species was not successful (Liu *et al.,* 2013).

PFGE band patterns, using a macrorestriction with *Apa*I enzyme, were obtained for isolates belonging to the *B. pumilus* group species phylogenetically assigned in this study (Fig. 5). Previous attempt to assess clonally structure of *B. pumilus* by PFGE were restricted to a few isolates (Ouoba *et al.,* 2004).

Two distinct genomic profiles (comprising ca. 19 to 20 bands), which shared a global similarity value of 66.7%, were obtained for *B. pumilus* isolates (Fig. 5a). The PFGE-type I_p corresponds to a clone contaminating medicine products of a pharmaceutical production unit. As expected, the three isolates belonging to this clone shared 100% of homology in the correspondent *rpoB* and *gyrB* sequences. PFGE-type $II_{p,}$ which shared a global similarity value of 66.7% with PFGE-type I_p corresponds to *B. pumilus* ATCC 14884.

A high genetic diversity was observed among *B. safensis* isolates (Fig 5b) (n=24; 64.3% of similarity in the DICE/UPGMA dendrogram), in which two major groups, arbitrarily designated as A (n=14, 72.3% of similarity) and B (n=9, 94.8% of similarity) were defined. These groups, which shared a global similarity value of 67.3%, were sub-divided into fourteen different pulsed-field types. PFGE-type V_s (of group A) comprised all animal isolates (Bs1, Bs2 and Bs3), obtained in different regions and years, which suggests the presence of a *B. safensis* clone highly adapted to a gastropod. This ecological specificity was also supported by *gyrB* and *rpoB* sequences analysis, which revealed 99.9% and 100% of similarity, respectively. PFGE-types II_s, VI_s, XI_s and XII_s encompassed isolates recovered from cosmetic products of different manufacturers and PFGE-types III_s, IV_s and VII_s comprised three isolates recovered from medicine products of a pharmaceutical production unit, also demonstrating the interest of this methodology to trace *B. safensis* contamination in industrial production lines. PFGE-types $XIII_s$ and XIV_s clustering into group B, enclosed nine isolates recovered by NASA from clean room assembly-facility surfaces, indicating the dissemination in this environment of a major clone (type $XIII_s$) to which belong *B. safensis* type strain. The remaining PFGE-types (I_s, $VIII_s$, IX_s and X_s), with a low similarity with the remainders, involved isolates from the PGPR and food contaminants. A more distantly related clone (PFGE type XV_s) was found in another food isolate.

In, figure 5c the clonal relationship of *B. altitudinis* isolates is shown. These isolates shared a 65.3% of genetic similarity and included 6 PFGE-types. PFGE-type I_a corresponds to a clone found in three medicine products of a pharmaceutical production unit, indicating a clonal contamination of a medicine production line. Congruently, these isolates shared by *rpoB* and *gyrB* sequences analysis 100% and 99.9% of homology, respectively. The remaining PFGE-types included isolates collected from other medicines contaminants, PGPR, food condiments products and the isolate associated with the foodborne poisoning outbreak (Ba26), which also presented a unique PFGE-type.

Thus, macrorestriction with *Apa*I enzyme followed by PFGE allowed the assessment of intra-species diversity of *B. pumilus* group species, depicting a high heterogeneity among *B. pumilus*, *B. safensis* and *B. altitudinis* isolates. Interestingly, our data also suggests a host clonal specificity supporting a great adaptability of *B. pumilus* members, which possibly justify the multiple ranges of activities formerly attributed to *B. pumilus*, and highlights the relevance of characterization at strain level of these species.

In conclusion, genomic characterization of a collection of isolates previously identified as *B. pumilus* revealed a highly heterogeneous population comprising different species (*B. pumilus, B. safensis* and *Bacillus altitudinis)* and clones. Moreover, we extend previous recognized niches and relevance of *B. safensis* and *Bacillus altitudinis.* Additionally, we also demonstrated that these species encompass a clonally diverse population, able to adapt to different hosts, which could justified the range of niches/hosts and activities previously attributed to *B. pumilus*.

Thus, this study contributed to unveil the population diversity, niches, and relevance of *B. pumilus, B. safensis and B. altitudinis,* which is of significance for understanding the ecology of *B. pumilus* group members and for the sectors where these microorganisms are intentionally used or can act as frequent contaminants.

Table1. Phylogenetic assignment and pulsotypes of *Bacillus pumilus* group isolates from diverse terrestrial sources.

Species		Isolate	Origin/Product	Year/ Location	GenBank accession numbers			PFGE-type	References
Previous identification	Phylogenetic assignment[1)]				16S rDNA gene	*gyrB* gene	*rpoB* gene		
B. pumilus	*B. pumilus*	Bp ATCC14884			JF749284	KC895462	KC895450	II_p	Reference strain
		Bp ATCC7061^T			EU138517	AY167869	AB353945	NA	Type-strain
		Bp7	Medicine´s contaminants (n=3)	2005/Portugal[1]	JN699029	JX183193	JX183164	I_p	Branquinho *et al.,* 2012
		Bp11		2005/Portugal[1]	JN699027	JX183196	JX183167		
		Bp15		2005/Portugal[1]	JN699025	JX183200	JX183171		
B. safensis	*B. safensis*	BsFO-36b^T	Clean-room/ air particulate (n=1)	1999/USA[6]	AF234854	AY167867	KC895451	$XIII_s$	Satomi *et al.,* 2006
		Bs32	Clean-room/entrance floor (n=1)	2001/USA[6]	AY167886	AY167877	KC895453		
		Bs42	Clean-room/anteroom (n=1)	2001/USA[6]	AY167884	AY167876	KC895454	Non typeable	
		Bs34	Clean-room/floor (n=2)	2001/USA[6]	AY167881	AY167873	KC895455	Non typeable	
		Bs35		2001/USA[6]	AY167880	AY167872	KC895456	XIV_s	
		Bs36	Clean-room/cabinet top (n=1)	2001/USA[6]	AY030327	AY167878	KC895452	$XIII_s$	
		Bs37	Clean-room/Mars Odyssey spacecraft surface (n=5)	2001/USA[6]	AF526907	KC895457	KC895445	$XIII_s$	
		Bs38		2001/USA[6]	AF526902	KC895458	KC895446		
		Bs39		2001/USA[6]	AF526898	KC895459	KC895447		
		Bs40		2001/USA[6]	AF526896	KC895460	KC895448		
		Bs41		2001/USA[6]	AF526895	KC895461	KC895449		
B. pumilus		Bs1	Animals Gastropods (n=3)	2004/Portugal[1]	JN699032	JX183188	JX183159	V_s	Branquinho *et al.,* 2012
		Bs2		2005/Portugal[1]	JX183146	JX183189	JX183160		
		Bs3		2007/Portugal[1]	JN699031	JX183190	JX183161		
		Bs13	Medicine´s contaminants (n=3)	2005/Portugal[1]	JX183149	JX183198	JX183169	VII_s	
		Bs16		2005/Portugal[1]	JN699024	JX183201	JX183172	III_s	
		Bs17		2005/Portugal[1]	JX183151	JX183202	JX183173	IV_s	
		Bs5	Cosmetic´s	2002/Portugal[1]	JN699030	JX183191	JX183162	XI_s	

B. pumilus	*B. safensis*	Bs18	contaminants (n=4)	2002/Portugal[1]	JN699023	JX183203	JX183174	VI_s	
		Bs19		2002/Portugal[1]	JN699022	JX183204	JX183175	XII_s	
		Bs27		2002/Portugal[1]	JN699021	JX183212	JX183183	II_s	
		Bs24	Food´s contaminants/salame (n=3)	2004/Italy[3]	JX183156	JX183209	JX183180	XV_s	Matarante *et al.,* 2004
		Bs25		2004/Italy[3]	JX183157	JX183210	JX183181	$VIII_s$	
		Bs33		2004/Italy[3]	KC895444	KC895463	JX183187	Non typeable	
		Bs22	Plant Growth Promoters (PGPR) (n=2)	1997/USA[4]	JX183154	JX183207	JX183178	IX_s	Jetiyanon 1997
		Bs23		1997/USA[4]	JX183155	JX183208	JX183179	I_s	
		Bs31	Food/beans (n=1)	2003/Africa[5]	KC895443	JX183215	JX183186	X_s	Ouoba *et al.,* 2004
	B. altitudinis	Ba6	Medicine´s contaminants (n=5)	2005/Portugal[1]	JX183147	JX183192	JX183163	I_a	Branquinho *et al.,* 2012
		Ba8		2005/Portugal[1]	JN699028	JX183194	JX183165		
		Ba9		2005/Portugal[1]	JX183148	JX183195	JX183166	III_a	
		Ba12		2005/Portugal[1]	JN699026	JX183197	JX183168	I_a	
		Ba14		2005/Portugal[1]	JX183150	JX183199	JX183170	II_a	
		Ba26	Food´s contaminants/rice (n=1)	2006/Norway[2]	JX183158	JX183211	JX183182	VI_a	From *et al.,* 2007
		Ba20	Plant Growth Promoters (PGPR) (n=2)	1996/USA[4]	JX183152	JX183205	JX183176	Non typeable	Wei *et al.,* 1996
		Ba21		1997/USA[4]	JX183153	JX183206	JX183177	V_a	Jetiyanon 1997
		Ba30	Food/beans (n=1)	2003/Africa[5]	KF060662	JX183214	JX183185	IV_a	Ouoba *et al.,* 2004

[1)]Species identification according to the phylogenetic affiliation obtained with *rpoB* and g*yrB* genes.

NA – not applicable.

Table 2. PCR primers sequences and amplification conditions for detection of 16S rRNA, *gyrB* and *rpoB* genes.

Gene	Primer name	Primer sequences 5´- 3´	PCR amplification				References
			Denaturation	Annealing	Cycles	Final extension	
16S rDNA	Seq_A Seq_B	AGAGTTTGATCHTGGYTYAGA ACGYTACCTTGTTACGACTTC	94ºC for 30 s	55ºC for 60 s	30	72ºC for 120s	Héritier *et al.*, 2003
β-subunit of DNA gyrase (*gyrB*)	UP-1S UP-2Sr	GAAGTCATCATGACCGTTCTGCAYGCNGG NGGNAARTTYGA AGCAGGGTACGGATGTGCGAGCCRTCNAC RTCNGCRTCNGTCAT	94ºC for 60 s	60ºC for 60 s	30	72ºC for 120s	Yamamoto *et al.*, 1995
β-subunit of RNA polymerase (*rpoB* gene)	$rpoB_{1\_fw}$ $rpoB_{1\_rv}$ $rpob_{2\_fw}$ $rpoB_{2\_rv}$ $rpoB_{3\_fw}$ $rpoB_{3\_rv}$ $rpoB_{4\_fw}$ $rpoB_{4\_rv}$	CAGAAGCTACGCACGCATAA GCGTCCAACATTTGCTAGGT CAACACGCTGGAAAAAGACA TTCTCGGCGTCACTTTACCT CACCAGAGGGTCCAAACATT GGTTGCGTAAAGCATCTTCC CATGAGTGAGCGCCTTGTAA CGTCTGCTTTCTTCGTTTCC	94ºC for 30 s	56ºC for 60 s	30	72ºC for 120s	This study

Table 3. Gene sequences of 16S *rRNA*, *gyrB* and *rpoB* for *Bacillus pumilus* group isolates deposited on GenBank and included in the phylogenetic analysis.

Species	Isolate	Origin	Region	GenBank accession numbers			References
				16S rDNA gene	*gyrB* gene	*rpoB* gene	
B. pumilus	Bp10m	Sediment	Pacific Ocean	JX680074	JX680151	JX679996	Liu *et al.,* 2013
	Bp42m		Indian Ocean	JX680106	JX680183	JX680028	
	Bp46m			JX680110	JX680187	JX680032	
	Bp53m		Indian Ocean	JX680117	JX680194	JX680039	
	Bp51m		Atlantic Ocean	JX680115	JX680192	JX680037	
	Bp66m			JX680129	JX680206	JX680051	
	Bp43m	Surface water	Pacific Ocean	JX680107	JX680184	JX680029	
	Bp44m		Pacific Ocean	JX680108	JX680185	JX680030	
	Bp67m	White shrimp	Shrimp farm	JX680130	JX680207	JX680052	
	Bp68m		Shrimp farm	JX680131	JX680208	JX680053	
	Bp69m	Aquaculture water	Shrimp farm	JX680132	JX680209	JX680054	
	Bp SAFR-032	Spacecraft Assembly Facility - NASA Jet Propulsion Laboratory	USA	NR074977	NC009848	CP000813	Gioia *et al.,* 2007
B. safensis	Bs30m	Sediment	South China Sea	JX680094	JX680171	JX680016	Liu *et al.,* 2013
	Bs35m			JX680099	JX680176	JX680021	
	Bs36m			JX680100	JX680177	JX680022	
	Bs37m			JX680101	JX680178	JX680023	
	Bs49m		Arctic Ocean	JX680113	JX680190	JX680035	
	Bs50m			JX680114	JX680191	JX680036	
	Bs64m		Bering Sea	JX680127	JX680204	JX680049	
	Bs45m	Surface water	Pacific Ocean	JX680109	JX680186	JX680031	
	Bs62m		Arctic Ocean	JX680126	JX680203	JX680048	
	Bs71m	Aquaculture water	Shrimp farm	JX680134	JX680211	JX680056	
	Bs60m	Intestinal tract contents of fish	Xiamen island	JX680124	JX680201	JX680046	
B. altitudinis	Ba3m	Sediment	Pacific Ocean	JX680068	JX680144	JX679989	
	Ba4m			JX680069	JX680145	JX679990	
	Ba6m			JX680070	JX680147	JX679992	
	Ba7m			JX680071	JX680148	JX679993	

B. altitudinis	Ba9m	*Sediment*	*Pacific Ocean*	JX680073	JX680150	JX679995	Liu *et al*., 2013
	Ba11m			JX680075	JX680152	JX679997	
	Ba12m			JX680076	JX680153	JX679998	
	Ba13m			JX680077	JX680154	JX679999	
	Ba14m			JX680078	JX680155	JX680000	
	Ba15m			JX680079	JX680156	JX680001	
	Ba20m		Indian Ocean	JX680084	JX680161	JX680006	
	Ba52m			JX680116	JX680193	JX680038	
	Ba47m		Atlantic Ocean	JX680111	JX680188	JX680033	
	Ba48m		Arctic Ocean	JX680112	JX680189	JX680034	
	Ba74m			JX680137	JX680214	JX680059	
	Ba8m		South China Sea	JX680072	JX680149	JX679994	
	Ba19m			JX680083	JX680160	JX680005	
	Ba28m			JX680092	JX680169	JX680014	
	Ba29m			JX680093	JX680170	JX680015	
	Ba31m			JX680095	JX680172	JX680017	
	Ba32m			JX680096	JX680173	JX680018	
	Ba33m			JX680097	JX680174	JX680019	
	Ba38m			JX680102	JX680179	JX680024	
	Ba39m			JX680103	JX680180	JX680025	
	Ba40m			JX680104	JX680181	JX680026	
	Ba17m	Surface water	Xiamen island	JX680081	JX680158	JX680003	
	Ba18m		Indian Ocean	JX680082	JX680159	JX680004	
	Ba24m		Yellow Sea	JX680088	JX680165	JX680010	
	Ba26m			JX680090	JX680167	JX680012	
	Ba25m		East China Sea	JX680089	JX680166	JX680011	
	Ba16m	Bottom water	Indian Ocean	JX680080	JX680157	JX680002	
	Ba27m		Pacific Ocean	JX680091	JX680168	JX680013	
	Ba21m		South China Sea	JX680085	JX680162	JX680007	
	Ba22m			JX680086	JX680163	JX680008	
	Ba23m			JX680087	JX680164	JX680009	
	Ba72m			JX680135	JX680212	JX680057	
	Ba75m	Aquaculture water	Shrimp farm	JX680138	JX680215	JX680060	
	Ba54m	Coral	Dongshan island	JX680118	JX680195	JX680040	
	Ba55m			JX680119	JX680196	JX680041	

B. altitudinis	Ba56m			JX680120	JX680197	JX680042	Liu *et al*., 2013
	Ba57m			JX680121	JX680198	JX680043	
	Ba58m			JX680122	JX680199	JX680044	
	Ba2m	Intestinal tract contents of fish	Xiamen island	JX680067	JX680143	JX679988	
	Ba61m			JX680125	JX680202	JX680047	
	Ba73m			JX680136	JX680213	JX680058	
	Ba76m	White shrimp	Shrimp farm	JX680139	JX680216	JX680061	
	Ba77m			JX680140	JX680217	JX680062	
	Ba78m			JX680141	JX680218	JX680063	
	Ba41KF2b[T]	High-elevation air sample	India	AJ831842	JX680219	JX680064	Shivaji *et al.,* 2006

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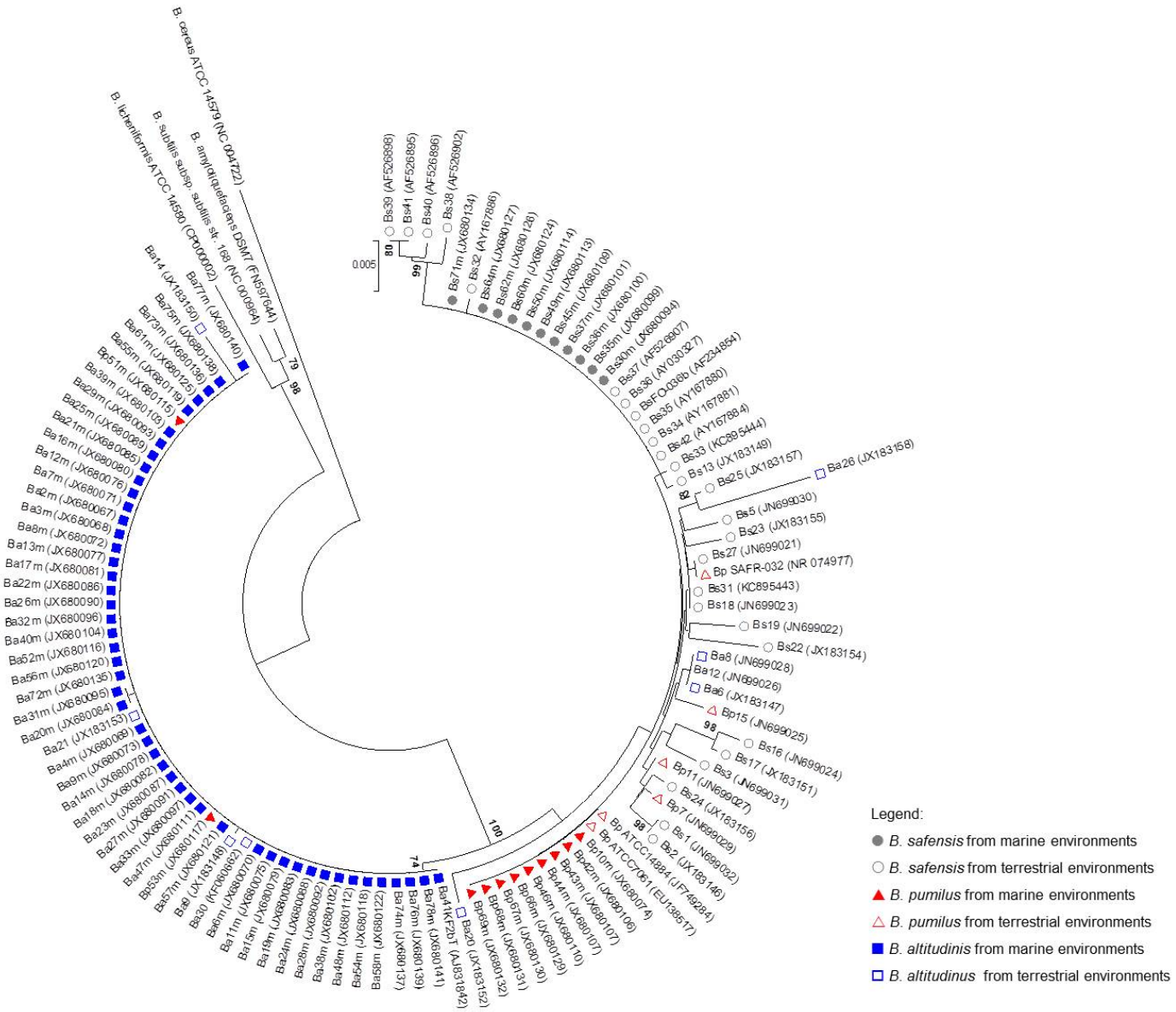
0.005
Legend:
B. safensis from marine environments
B. safensis from terrestrial environments
B. pumilus from marine environments
B. pumilus from terrestrial environments
B. altitudinis from marine environments
B. altitudinus from terrestrial environments

Figure 1. Neighbour-joining (NJ) tree based on the 16S rRNA gene sequences comparison, showing the relationship of *B. pumilus* group members, namely *B. safensis*, *B. pumilus* and *B. altitudinis*. Genetic distances were constructed using Kimura´s 2-parameter model. Numbers at branch points indicated bootstrap percentages (1000 replications) from NJ analysis and only values greater than 70% were shown. *Bacillus subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* DSM7^T, *B. licheniformis* ATCC 14580^T and *B. cereus* ATCC 14579^T were used as outgroups. Accession numbers were given in parentheses. Symbols represent: *B. pumilus* from terrestrial (△) and marine (▲) settings; *B. safensis* from terrestrial (○) and marine (●) settings and *B. altitudinis* from terrestrial (□) and marine (■) settings. Bar: genetic distance of 0.005.

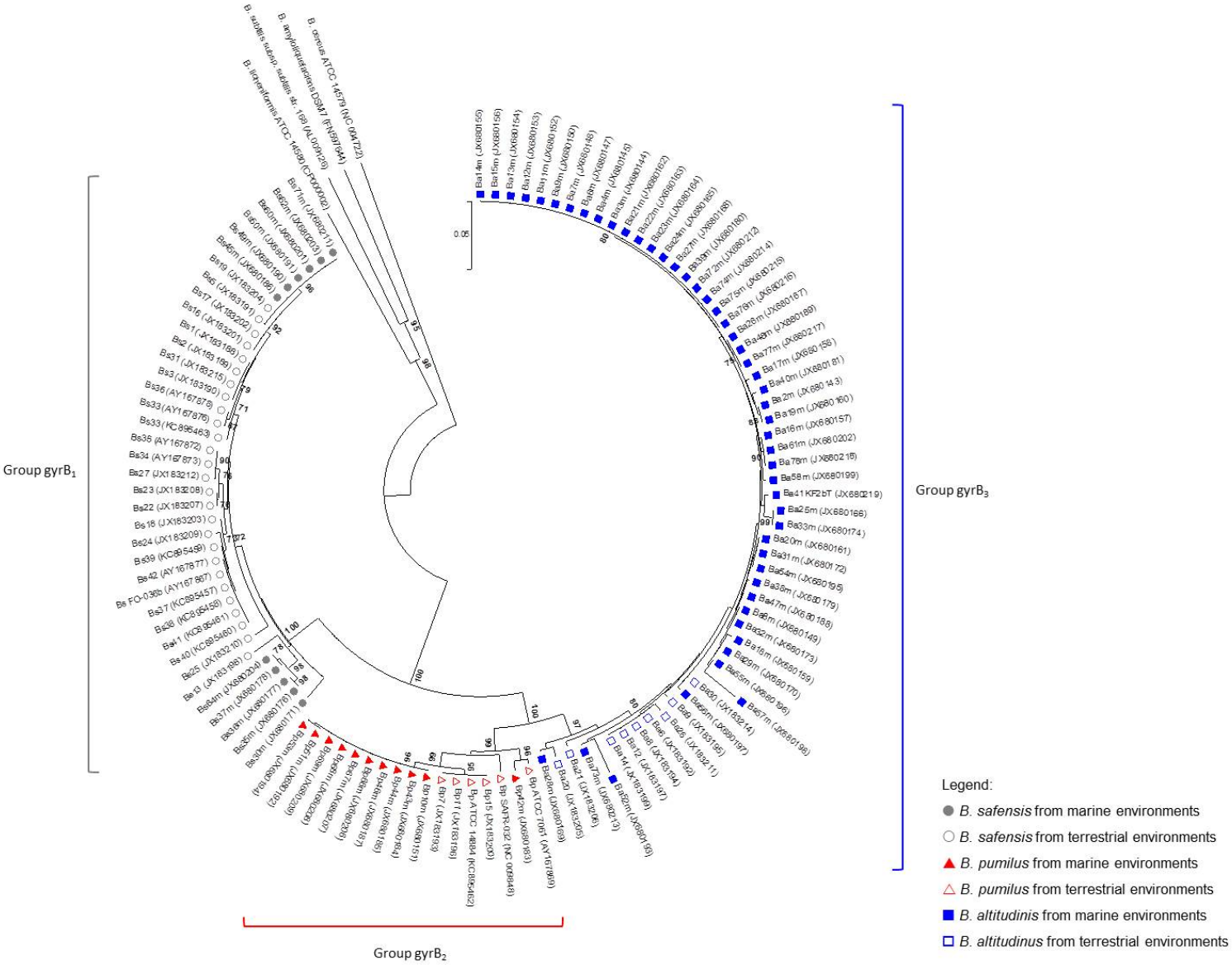
Group gyrB1
Group gyrB2
Group gyrB3
Legend:
B. safensis from marine environments
B. safensis from terrestrial environments
B. pumilus from marine environments
B. pumilus from terrestrial environments
B. altitudinis from marine environments
B. altitudinus from terrestrial environments

Figure 3. Neighbour-joining (NJ) tree based on the *gyrB* gene sequences comparison, showing the relationship of *B. pumilus* group members, namely *B. safensis*, *B. pumilus* and *B. altitudinis*. Genetic distances were constructed using Kimura´s 2-parameter model. Numbers at branch points indicated bootstrap percentages (1000 replications) from NJ analysis and only values greater than 70% were shown. *Bacillus subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* DSM7^T, *B. licheniformis* ATCC 14580^T and *B. cereus* ATCC 14579^T were used as outgroups. Accession numbers were given in parentheses. Symbols represent: *B. pumilus* from terrestrial (△) and marine (▲) settings; *B. safensis* from terrestrial (○) and marine (●) settings and *B. altitudinis* from terrestrial (□) and marine (■) settings. Bar: genetic distance of 0.05.

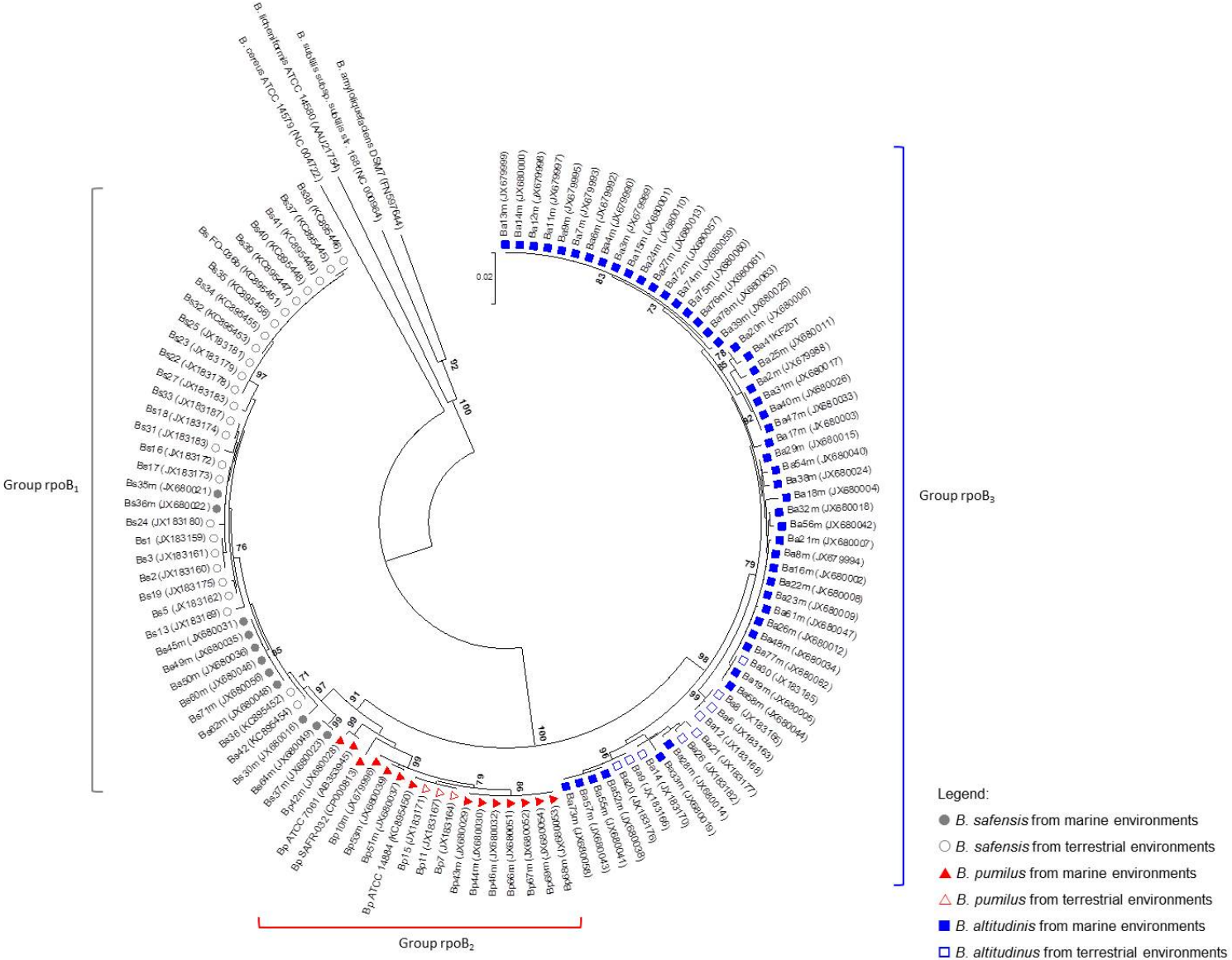
Group rpoB1
Group rpoB2
Group rpoB3
0.02
Legend:
B. safensis from marine environments
B. safensis from terrestrial environments
B. pumilus from marine environments
B. pumilus from terrestrial environments
B. altitudinis from marine environments
B. altitudinus from terrestrial environments

Figure 3. Neighbour-joining (NJ) tree based on the hypervariable region of *rpoB* gene sequences comparison, showing the relationship of *B. pumilus* group members, namely *B. safensis*, *B. pumilus* and *B. altitudinis*. Genetic distances were constructed using Kimura´s 2-parameter model. Numbers at branch points indicated bootstrap percentages (1000 replications) from NJ analysis and only values greater than 70% were shown. *Bacillus subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* DSM7^{T}, *B. licheniformis* ATCC 14580^{T} and *B. cereus* ATCC 14579^{T} were used as outgroups. Accession numbers were given in parentheses. Symbols represent: *B. pumilus* from terrestrial (△) and marine (▲) settings; *B. safensis* from terrestrial (○) and marine (●) settings and *B. altitudinis* from terrestrial (□) and marine (■) settings. Bar: genetic distance of 0.02.

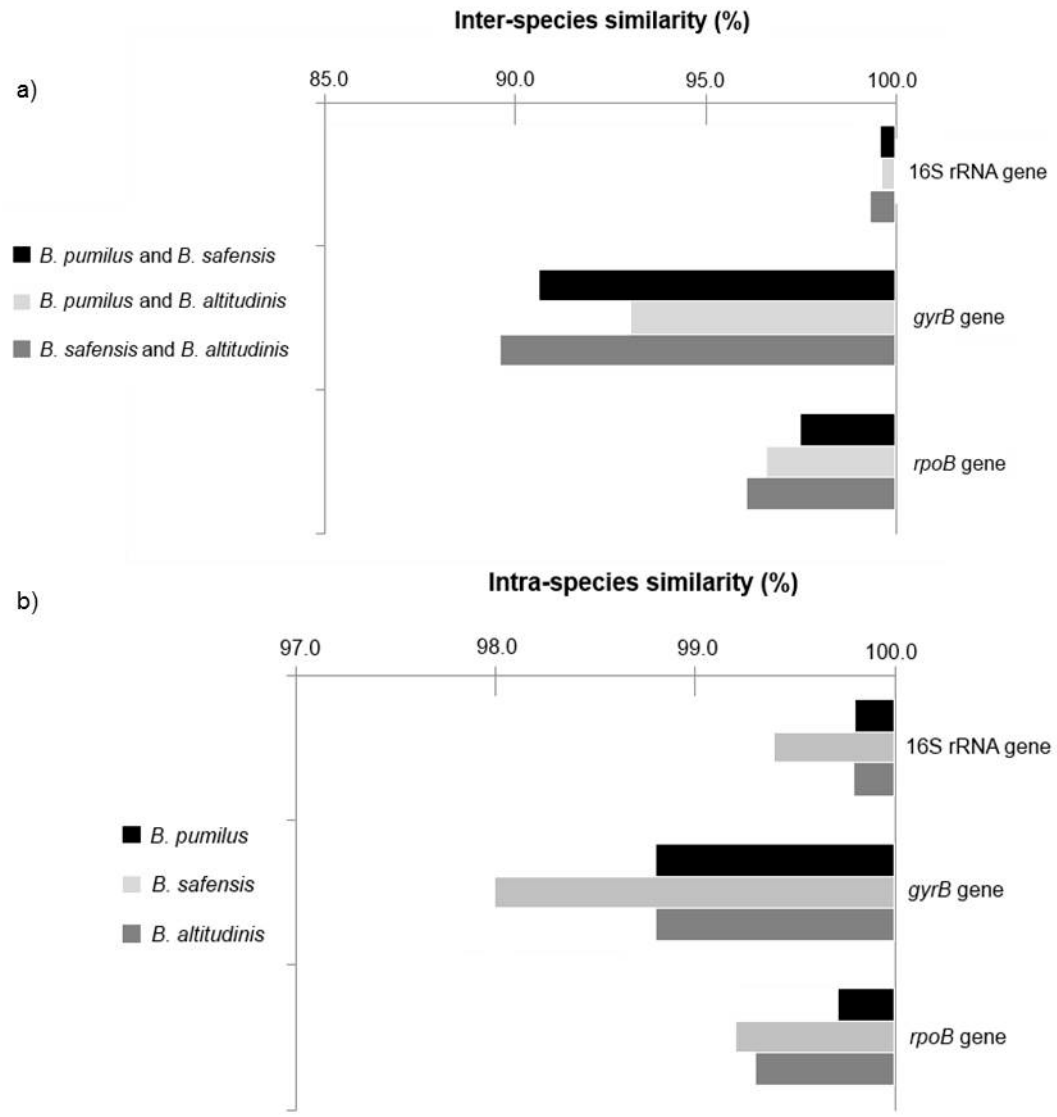

Figure 4. a) Inter- and b) intra-species similarity ranges of 16S rRNA, *gyrB* and *rpoB* genes in *Bacillus pumilus* group members.

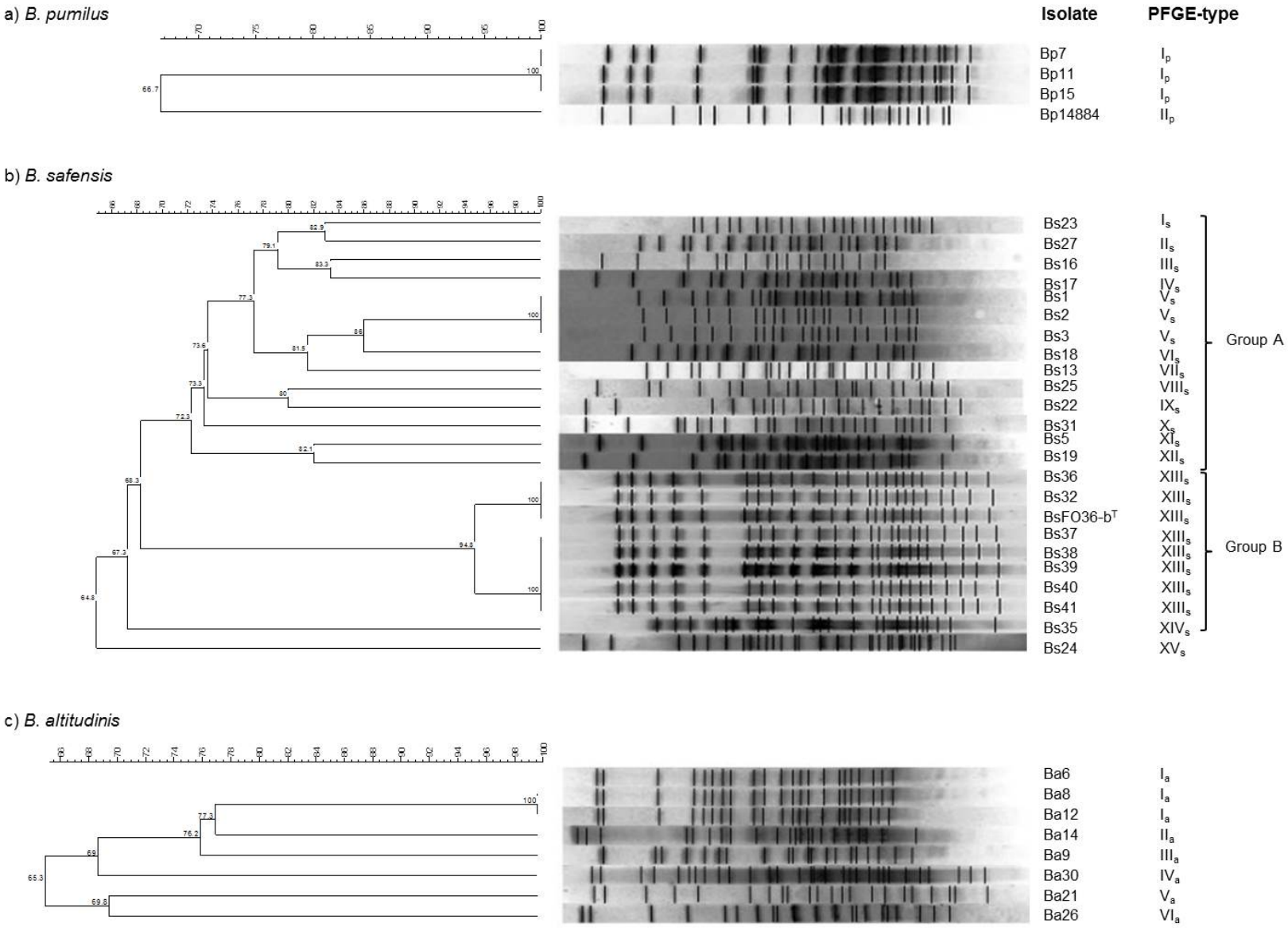
a) B. pumilus
Isolate
PFGE-type
Bp7 Ip
Bp11 Ip
Bp15 Ip
Bp14884 IIp
b) B. safensis
Bs23 Is
Bs27 IIs
Bs16 IIIs
Bs17 IVs
Bs1 Vs
Bs2 Vs
Bs3 Vs
Bs18 VIs
Bs13 VIIs
Bs25 VIIIs
Bs22 IXs
Bs31 Xs
Bs5 XIs
Bs19 XIIs
Bs36 XIIIs
Bs32 XIIIs
BsFO36-bT XIIIs
Bs37 XIIIs
Bs38 XIIIs
Bs39 XIIIs
Bs40 XIIIs
Bs41 XIIIs
Bs35 XIVs
Bs24 XVs
Group A
Group B
c) B. altitudinis
Ba6 Ia
Ba8 Ia
Ba12 Ia
Ba14 IIa
Ba9 IIIa
Ba30 IVa
Ba21 Va
Ba26 VIa

Figure 5. Dendrogram resulting from cluster analysis of PFGE fingerprints of *Apa*I-digested DNA from typeable *B. pumilus* (a), *B. safensis* (b) and *B. altitudinis* (c) isolates. First and second columns (right) represented the isolate name and PFGE-types, respectively. Similarity values were achieved using UPGMA method. Band lines were automatically generated by InfoQuest software to better elucidate its position in the gel.

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Supporting Information

Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a highly heterogeneous population of different closely related species and clones

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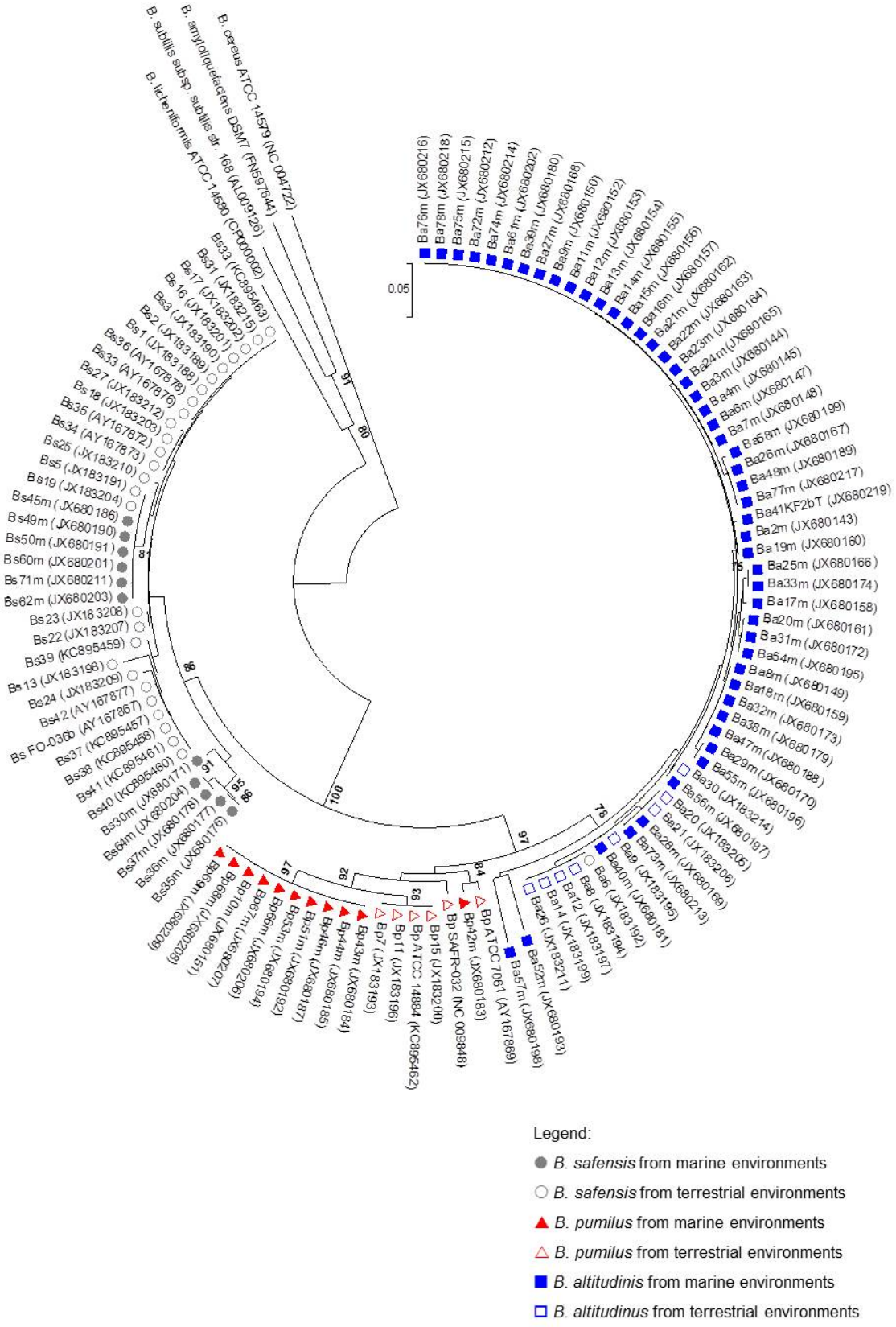
Running title: Species and clonal diversity within *Bacillus pumilus*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91
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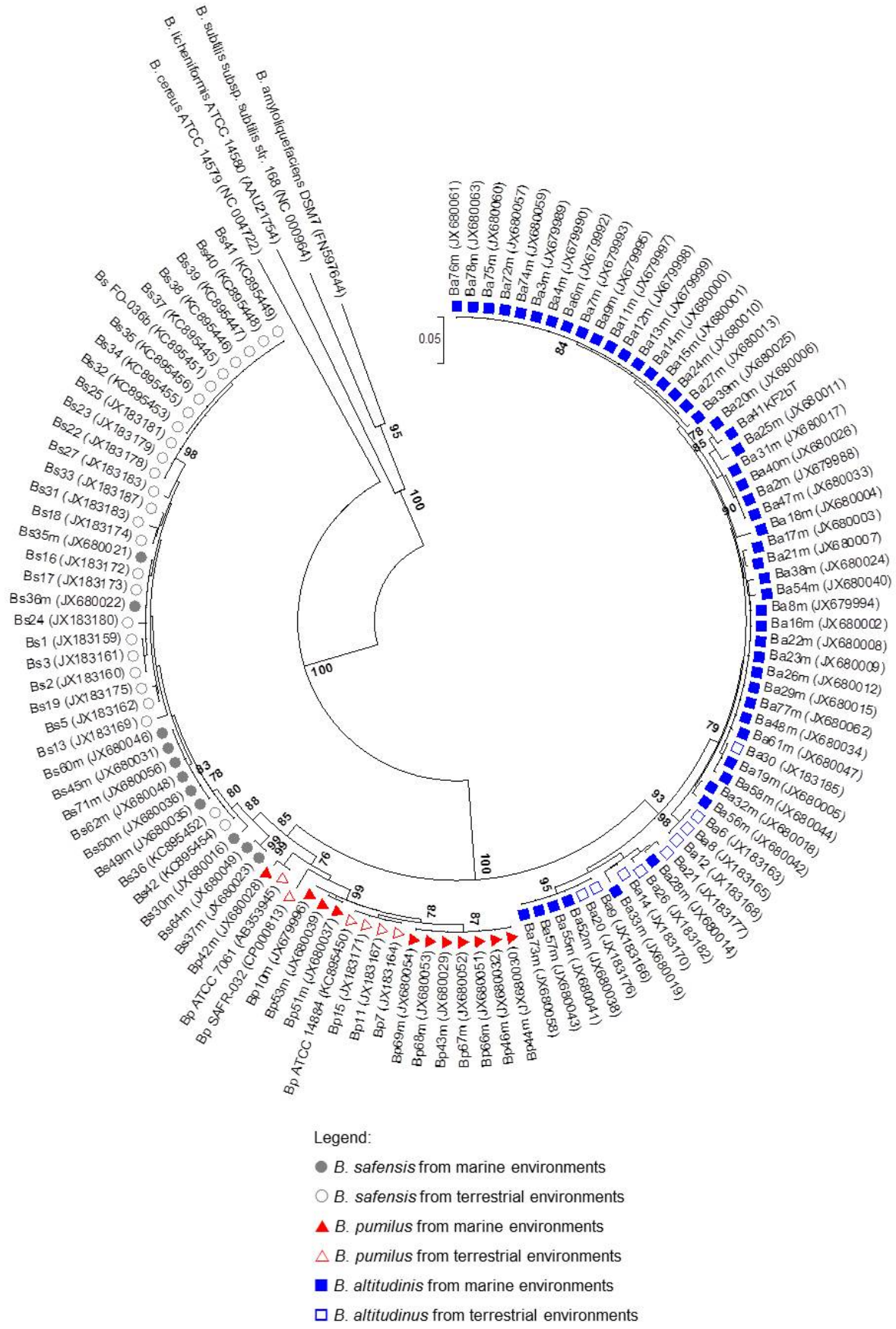
86
91
95
98
97
92
93
84
97
78
76
Legend:
B. safensis from marine environments
B. safensis from terrestrial environments
B. pumilus from marine environments
B. pumilus from terrestrial environments
B. altitudinis from marine environments
B. altitudinus from terrestrial environments

Figure S1. Phylogenetic tree inferred from GyrB protein sequences in *B. pumilus* group members. Genetic distances were constructed using Jones-Taylor-Thornton (JTT) model. Numbers at branch points indicate bootstrap percentages from Neighbor-Joining method analysis and only values greater than 70% were shown. *B. cereus* was used as the outgroup taxon. Symbols represent: *B. pumilus* from terrestrial (△) and marine (▲) settings; *B. safensis* from terrestrial (○) and marine (●) settings and *B. altitudinis* from terrestrial (□) and marine (■) settings. Bar: genetic distance of 0.05.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Legend:
B. safensis from marine environments
B. safensis from terrestrial environments
B. pumilus from marine environments
B. pumilus from terrestrial environments
B. altitudinis from marine environments
B. altitudinus from terrestrial environments

Figure S2. Phylogenetic tree inferred from RpoB protein sequences (based on hypervariable region of the *rpoB* gene) in *B. pumilus* group members. Genetic distances were constructed using Jones-Taylor-Thornton (JTT) model. Numbers at branch points indicate bootstrap percentages from Neighbor-Joining method analysis and only values greater than 70% were shown. *B. cereus* was used as the outgroup taxon. Symbols represent: *B. pumilus* from terrestrial (△) and marine (▲) settings; *B. safensis* from terrestrial (○) and marine (●) settings and *B. altitudinis* from terrestrial (□) and marine (■) settings. Bar: genetic distance of 0.05.

2.2. *Bacillus invictus* sp. nov., isolated from healthy products in Portugal

Publications:

***Bacillus invictus* sp. nov., isolated from healthy products in Portugal**

During a study adressing *Bacillus* spp. diversity, we uncover the presence of a different species closely related with members of *B. pumilus* group in contaminants of healthy products.The prediction of a new species is supported by DNA-DNA hybridization studies, *rpoB* and *gyrB* genes homologies, Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF/ICMS) and Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) analysis, reinforced by chemometric analysis (PCA) of respectively spectral data.

Bacillus invictus sp. nov., isolated from healthy products in Portugal

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Running title: *Bacillus invictus* sp. nov.

Keywords: *Bacillus invictus* sp. nov., *gyrB* gene analysis, *rpoB* gene analysis, polyphasic approach, MALDI-TOF/MS, FTIR-ATR

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Manuscript in preparation

Abstract

Three Gram-positive, rod-shaped endospore-forming *Bacillus* isolates Bi.$_{FFUP1}$, Bi.$_{FFUP2}$, and Bi.$_{FFUP3}$ recovered in Portugal from health´s products contaminants were subjected to a polyphasic study and comparison with *Bacillus pumilus*, *Bacillus safensis, Bacillus altitudinis* and *Bacillus xiamenensis* the phenotypically and genotypically closest species.

Acid production from cellobiose, glucose and mannose and absence of acid production when D-arabinose, erythritol, inositol, maltose, mannitol, raffinose, rhamnose, sorbitol, starch and L-tryptophan is tested, discriminate these new isolates from their closely related ones. Additionally, a significant different protein and carbohydrate signature was evidenced by spectroscopic techniques, MALDI-TOF/MS and FTIR-ATR. PCA (principal component analysis) of this spectral data clearly delineated the novel species isolates.

The quinone system is MK-7 and major polar lipids are diphosphatidylglycerol, an unidentified phospholipid and an unidentified glycolipid. Strain Bi.$_{FFUP1}^{T}$ showed ≥99% 16S rRNA gene similarity with *B. safensis* FO-036b^{T}, *B. pumilus (*ATCC 14884 and ATCC 7061^{T}), *B. altitudinis* 41KF2b^{T} and *B. xiamenensis* HYC-10^{T} Differences in strain Bi.$_{FFUP1}^{T}$ *gyrB* and *rpoB* sequences together with their phylogenetic tree topology also supported the delineation of a new species. Moreover, only 49-50% DNA relatedness (DDH studies) was observed between Bi.$_{FFUP1}$ and *B. safensis* FO-036b^{T} and 61.90% with Bi.$_{FFUP1}$ *a*nd *B. altitudinis* 41KF2b^{T}. Variable DDH values were obtained when Bi.$_{FFUP1}$ was compared with *B. pumilus* ATCC 14884 (39.75% ±0.35) and ATCC 7061^{T} (69.40%±2), despite the genotypic and phenotypic similarity of these two *B. pumilus* strains, highlighting the ambiguities in the taxonomic grouping based on a strict DDH cut-off.

Therefore, the three isolates represent a novel species of *Bacillus* genus, for which the name *Bacillus invictus* sp. nov. is proposed with strain Bi.$_{FFUP1}$ as the type strain.

Members of the *Bacillus* group, currently including near two hundred species (Parte, 2013), are well known to withstand resistance to heat, radiation, chemical oxidizing agents and desiccation, due to its spore production, which indeed represent the crucial condition to facilitate their adaptation into different ecological niches (Stackebrandt *et al.,* 2008). It is this resistance to acute stress factors that make them difficult to eliminate from clean room environments and, consequently, representing a frequent cause of significant contamination of many industrial products, including pharmaceuticals. Recently, National Aeronautics and Space Administration (NASA) have reported *B. safensis* and *B. pumilus* (Satomi *et al*., 2006), as some of the major contaminants in spacecraft and associated clean rooms assembly-facility surfaces. Taxonomically, *Bacillus pumilus* group species belong to RNA group I and encompass *B. pumilus*, the first species described, *Bacillus safensis, Bacillus altitudinis, Bacillus stratosphericus, Bacillus altitudinis* and the more recently species discover *Bacillus xiamenensis* (Satomi *et al.,* 2006; Liu *et al.,* 2013; Lai *et al.,* 2014).

During a study assessing the diversity of *Bacillus* spp. isolates recovered from terrestrial sources, we disclosed the presence as contaminant of healthy products of a different species closely related with members of *B. pumilus* group. Thus, in the present study, chemotaxonomic characteristics, which included carbohydrate and amino acid metabolic profiles, whole-cell fatty acid methyl ester, respiratory quinones, polar lipids, protein and carbohydrate content, together with phylogenetic features derived from DNA-DNA hybridization (DDH) studies, 16S rRNA, *rpoB* and *gyrB* gene sequences were assessed in our isolates and compared with ones from *B. safensis, B. pumilus, B. altitudinis, B. xiamenensis*, the available closest phenotypic and genotypic species, supporting the description of a novel species, which is of relevance in food and pharmaceutical quality control and for infection control purposes.

Three *Bacillus* sp. isolates were recovered from healthy products in Portugal, designated Bi.$_{FFUP1}$, Bi.$_{FFUP2}$, and Bi.$_{FFUP3,}$ (n=3), being Bi.$_{FFUP1}^{T}$ classified as the type-strain. All strains were maintained on Trypticase Soy Agar (TSA; Sigma-Aldrich) for short-term storage and in Tryptic Soy Broth (TSB; Sigma-Aldrich) supplemented with 20% (v/v) glycerol at -80 ºC for long-term storage. Liquid cultures were grown in TSB at 37 ºC for 24 h. In addittion, the most phenotypically and genotypically closest species, *B. safensis* FO-036b^{T}*, B. pumilus* ATCC 14884 and 7061^{T}*, B. altitudinis* 41KF2b^{T} and *B. xiamenensis* HYC-10^{T} were obtained from the respective culture collections and cultivated at 37 ºC in TSA,

which were used as references for most of the comparative analysis applied during the study.

In order to phenotypically characterize the isolates, standard phenotypic tests were performed. Gram staining was determined using the bioMérieux Gram Stain kit according to the manufacturer´s instruction. Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl-p-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution. Growth at different concentrations of salt was determined in salt-free TSB supplemented with 0, 5.0, 7.5, 10.0, 12.5 and 15.0% (w/v) NaCl. The pH range of growth was determined on TSA at pH values ranging from 6.0 to 8.0 for 3 weeks. Optimum and limiting temperatures were determined by growing cells on TSA at 4, 25, 37, 50 and 60 ºC. Carbohydrate and amino acid metabolic profiles were obtained with a commercial BBL Crystal Gram Positive ID Kit (Becton Dickinson) and API 50CH (bioMérieux), which were interpreted according to the manufacturer's instructions.

For quantitative analysis of whole-cell fatty acid methyl ester profiles, novel isolates were cultivated on TSA at 30 ºC for 24 h. Preparation and analysis of fatty acid methyl esters were conducted according to the instructions of the Sherlock® Microbial Identification System (MIS) (Microbial Identification System, Sherlock). Moreover, quinones and polar lipids were harvest from *Bacillus* sp. biomass grown in PYE broth and extracted and analysed according to the integrated procedure described by Tindall (1990 a and b) and Altenburger *et al*. (1996). HPLC analysis was carried out using the HPLC apparatus described by Stolz *et al*. (2007). Further, polar lipids were separated by two-dimensional silica gel thin layer chromatography and then identified according to a previously described method (Tindall *et al*., 2007).

The snapshot of different protein compositions detected by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) was acquired by a MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, AB SCIEX) operating in linear positive mode. Each spectrum was the accumulated sum of at least 2000 laser shots for *m/z* 2500-12000 due to the good reproducibility of the spectral profile in that mass range. All the spectra were externally calibrated using a commercial mixture of angiotensin I, ACTH and insulin (AB SCIEX) and analyzed with the Data Explorer software (Version 4.6, AB SCIEX). *Bacillus* sp. isolates were grown under aerobic conditions on LB agar (Merck) for 24 h at 37 °C. Cells were harvested by transferring the equivalent of three full blue plastic loops (≈ 30 µl) from each agar plate into 20 µl of sterile water and bacterial material resuspended by vortexing. Sample inactivation was carried

out applying the modified trifluoroacetic acid (TFA) (Sigma-Aldrich) inactivation protocol (Lasch *et al*., 2008). Subsequent supernatant filtration (0.22 µm) was carried out. For MALDI-TOF/MS experiments, 2 µl of the filtrated microbial dilution were mixed with 2 µl of a 12-mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich) solution in 100% ACN and 0.3% TFA. 1 µl of the mixture was spotted onto a stainless steel MALDI sample plate and allowed to dry at room temperature. For each isolate, two biological replicates (obtained from two different agar plates) were carried out and the mean spectra were considered for the analysis. Mass spectra were baseline corrected, noise-filtered and *m/z* values were extracted from the raw experimental mass spectra that included all the mass peaks with a relative signal to noise (S/N) ratio intensity above 2.

In addition, whole cell content of the bacterial isolates studied was analyzed by Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR). Spectra were acquired using a FTIR System spectrophotometer (PerkinElmer Spectrum BX) in the ATR mode with a PIKE Technologies Gladi ATR accessory from 4000-600 cm^{-1} and a resolution of 4 cm^{-1} and 32 scan co-additions. Isolates were grown for 37 ºC at 17 h, on Mueller Hinton Agar (MH; BioMérieux) and colonies were directly applied in the ATR crystal and dried in a thin film. For each isolate, six spectra were acquired, corresponding to three biological replicates (obtained from two different agar plates) and two instrumental replicates (obtained from the same agar plates) and the mean spectra was considered in the analysis.

Chemometric analysis of MALDI-TOF/MS and FTIR-ATR spectra were performed in Matlab version 6.5 release 13 (MathWorks) and the PLS Toolbox version 3.5 for Matlab (Eigenvector Research). FTIR-ATR spectra were processed with standard normal variate (SNV) (Næs *et al*., 2002) followed by the application of a Savitzky-Golay filter (9 smoothing points, 2^{nd} order polynomial and second derivative) (Savitzky & Golay, 1964) and mean-centred. MALDI-TOF/MS spectra were mean-centred. Spectra were analysed by principal component analysis (PCA) (Hotelling, 1933) considering the whole spectra for MALDI-TOF/MS and the carbohydrates region (1200-900 cm^{-1}) for FTIR-ATR. Carbohydrates region was chosen due to its higher variability among the isolates.

Comparative phylogenetic analyses were also performed with the newly described *Bacillus* isolates and reference strains. The 16S rRNA gene amplification was conducted using the universal primer pair Seq_A and Seq_B (Héritier *et al.,* 2003). β-subunit of DNA gyrase (*gyrB* gene) was amplified as described previously (Yamamoto *et al*., 1995) using the degenerate primers UP-1S and UP-2Sr. β-subunit of RNA polymerase (*rpoB* gene) was amplified using combinations ($rpoB_{1\_fw}$ and $rpoB_{1\_rv}$, $rpob_{2\_fw}$ and $rpoB_{2\_rv}$, $rpoB_{3\_fw}$

and $rpoB_{3\_rv}$, $rpoB_{4\_fw}$, and $rpoB_{4\_rv}$) of primer pairs, which are shown in Supplementary Table S2. The identity of a given PCR product was verified by sequencing analysis. Sequences obtained were compared to 16S rRNA, *gyrB* and *rpoB* gene sequences of *B. safensis* FO-036b*, B. pumilus* ATCC 14884 and 7061*, B. altitudinis* 41KF2b and *B. xiamenensis* HYC-10, available on GenBank database, according to Table 2, using the BLAST search. Nucleotide sequences were aligned and species similarity score for each gene were generated using MEGA version 5.2.2 (http://www.megasoftware.net/) (Tamura *et al.,* 2011). Phylogenetic trees were constructed from the alignment similarity scores using neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-likelihood (ML) (Felsenstein, 1981) algorithms. During the analysis of alignments, genetic distances were estimated using Jukes-Cantor model (Jukes & Cantor, 1969), where all substitutions were included in pairwise distance calculations. The reliability of internal branches was assessed from bootstrap based on 1000 resamplings (Felsenstein, 1985). To establish phylogenetic analysis, *rpoB* and *gyrB* sequences described in *B. subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* $DSM7^T$*, B. licheniformis* ATCC 14580^T and *B. cereus* ATCC 14579^T were used as outgroups. Nucleotide sequences of the 16S rRNA, *rpoB*, *gyrB* and genes obtained in this study from $Bi._{FFUP1}$, $Bi._{FFUP2}$, and $Bi._{FFUP3}$ isolates were deposited into GenBank and assigned with NCBI accession numbers (Table 2).

Accordingly Adékambi *et al*. (2009), the total genomic G+C content can be inferred from the G+C content of universally conserved genes, with a strong correlation (coefficient of determination ($\mathbf{r^2}$) = 0.97) between the G+C content of the *rpoB* gene and that of the correspondent genome. Thus, the genomic G+C content (GCg) of $Bi._{FFUP1}$ was estimated by the *rpoB* gene GC content (GCr) using the following formula: GCg = 1.2065 x GCr – 11.495 (Adékambi *et al*., 2009).

For DNA-DNA hybridization studies, cells were disrupted using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and the DNA in the crude lysate purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA-DNA hybridization was conducted as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in situ* temperature probe.

The novel isolates were Gram-positive rods, aerobic, motile and contained terminally/ subterminally located spores, similar to their closely related species *B. safensis, B.*

pumilus, B. altitudinis and *B. xiamenensis*. When grown at 37 ºC for 24 h on TSA, colonies are round, smooth, yellowish in color, have irregular margins and were approximately 2.8 ±0.2mm in diameter. The cells were oxidase- and catalase-positive. Growth occurred at 25 ºC, 37 ºC and 50 ºC but not at 4 ºC or 60 ºC. Growth occurred in the range 0-10% (w/v) NaCl and at pH 6.0–8.0. Phenotypic details derived from metabolic tests obtained with BBL Crystal Gram Positive ID Kit and API 50 CH were detailed in species description and in supplementary Table S1. Acid production from cellobiose, glucose and mannose and absence of acid production when D-arabinose, erythritol, inositol, maltose, mannitol, raffinose, rhamnose, sorbitol, starch and L-tryptophan is tested, allowed the discrimination of the novel isolates from the related species (Table 1).

The major fatty acids profiles of novel isolates were $C_{15:0}$ iso (41.3%), $C_{15:0}$ anteiso (35.3%), $C_{17:0}$ iso (4.9%) and $C_{17:0}$ anteiso (6.6%), similar to previously described to their closed species (Table 3).

Moreover, quinones system consisted predominantly of menaquinone MK-7 (99.4%), which is consistent with members of *Bacillus* genus (Lai *et al.*, 2014), and also traces of MK-6 (0.3%) and MK-8 (0.2%).

Related to the polar lipid profile (Figure 5) diphosphatIdylglycerol (DPG), an unidentified phospholipid (PL1) and an unidentified glycolipid (GL2) were detected. Furthermore, moderate amounts of phosphatidylglycerol (PG), another unidentified glycolipid (GL1) and three lipids (L1-3) not containing a sugar, phosphate or amino residue were detected. In addition some other minor lipids were observed, including a third unidentified glycolipid (GL3), an aminolipid (AL1) and two lipids (L4, L5), not containing a sugar, phosphate or amino residue. However, GL2 showed a chromatographic motility similar to a glycolipid detected in *Bacillus subtilis* and considered to be *β*-gentiobiosyldiacylglycerol (Kämpfer *et al*., 2006).

Mass spectra of whole bacterial cells provide a snapshot of the bacterial protein composition and have been widely used to achieve specific biomarkers of closely related species (Šedo *et al*., 2011). Spectral profiles comparison of *B. pumilus* ATCC 7061^T, *B. safensis* FO-36b^T and Bi.$_{FFUP1}$ showed the presence of several species-specific mass peaks (Figure 1a, marked with *). Peaks with m/z values of 6100 was specific for Bi.$_{FFUP1}$; 3707, 4414, 5290, 5575, 6124, 6798 for *B. safensis* FO-36b^T and 3853, 4189, 4507, 4693, 5556, 5933, 6414, 6622, 6941, 7509, 8378, 9394, 11120 for *B. pumilus* ATCC 7061^T.

A PCA of all isolates (*B. pumilus* ATCC 14884 and 7061^T, *B. safensis* FO-036b^T and Bi.$_{FFUP1}$, Bi.$_{FFUP2}$, and Bi.$_{FFUP3}$) clearly revealed the presence of three distinct clusters (Figure 1b), comprising each one a different species. Moreover, from this analysis *B. pumilus* and *B. safensis* were discriminated from Bi.$_{FFUP1}$ in the first principal component (PC1), which accounts for 72.6% of the spectral variability, meaning that Bi.$_{FFUP1}$ was the most distinct species presented. Further studies comprising mass spectra's of *B. altitudinis* and *B. xiamenensis* should also be performed.

Bacterial infrared spectra of the whole bacterial cell content have already demonstrated great potential for bacterial identification at different taxonomic levels (Kuhm *et al*., 2009; Sousa *et al*., 2012). Whereas other methods require the use of specific marker to allow the bacterial differentiation, the infrared analysis provides an entire range of molecular information on the bacteria of interest. Spectra profiles of the type strains *B. pumilus* ATCC 7061^T, *B. safensis* FO-036b^T and Bi.$_{FFUP1}$ were also clearly distinct, considering the carbohydrates region comprised between 1200-900cm^{-1} (Figure 2a), which evidenced a different sugar composition among the three species. Furthermore and similarly to the MALDI-TOF/MS analysis, PCA (Figure 2b) of FTIR-ATR spectra revealed three perfectly individualized groups, each one containing a different species. The first principal component (PC1) allowed the discrimination of Bi.$_{FFUP1}$ from *B. pumilus* and *B. safensis* and accounted for 83.0% of the spectral variability. These results support Bi.$_{FFUP1}$ as a different species and also as the species more distantly related, when *B. pumilus* and *B. safensis* were compared. Similar to MALDI-TOF/MS approach, further studies comprising infrared spectra of the whole bacterial cell content of *B. altitudinis* and *B. xiamenensis* should also be performed

Phylogenetic analysis of 16S rRNA gene sequences grouped the three Bi.$_{FFUP1}$, Bi.$_{FFUP2}$ and Bi.$_{FFUP3}$ isolates close to *B. pumilus* ATCC 14884 and 7061^T, *B. safensis* FO-036b^T, *B. altitudinis* 41KF2b ^{T} and *B. xiamenensis* HYC-10^T with ≥99% of identity. Since limitations of 16S rRNA sequences to decipher relationships at species level are recognized, in which strains with >99% 16S rRNA sequences similarity may not belong to the same species (Wang *et al*., 2001), comparative *rpoB* and *gyrB* gene sequence analyses were carried out.

The isolates Bi.$_{FFUP1}$, Bi.$_{FFUP2}$ and Bi.$_{FFUP3}$ (revealing 99.8% of similarity among their *rpoB* gene sequence) shared 96.9%, 97.1%, 96.2%, 98.5% and 97.9% in *rpoB* sequence similarity with *B. pumilus* ATCC 14884 and 7061^T and *B. safensis* FO-036b^T, *B. altitudinis* 41KF2b^T and *B. xiamenensis* HYC-10^T, respectively. In addition, a 92.7%, 92.9%, 90.8%,

98.5% and 92.1% of similarity was achieved in *gyrB* gene sequence analysis between Bi.$_{FFUP1}$, Bi.$_{FFUP2}$ and Bi.$_{FFUP3}$ (sharing 100% of similarity among them) and *B. pumilus* ATCC 14884 and 7061^T and *B. safensis* FO-036b^T, *, B. altitudinis* 41KF2b^T and *B. xiamenensis* HYC-10^T, respectively. Moreover, these *rpoB* and *gyrB* genes sequences comparison were supported by a bootstrap value of >78% (Figure 3 and 4).

Thus, *rpoB* and *gyrB* genes sequence-based phylogenetic topology proved to be more discriminative than 16S rRNA gene sequences, also supporting the high degree of polymorphism recognized to these genes, grouping these strains monophyletically in a cluster separate from *B. pumilus* ATCC 14884 and 7061^T and *B. safensis* FO-036b^T, *B. altitudinis* 41KF2b^T and *B. xiamenensis* HYC-10^T representatives.

The DNA G+C content for Bi.$_{FFUP1}$ was 41 mol%.

DNA hybridization analyses was carried out to evaluate the genomic DNA-DNA relatedness between strain Bi.$_{FFUP1}$ and the most closely related reference strains *B. pumilus* ATCC 14884 and 7061^T, *B. safensis* FO-036b^T and *B. altitudinis* 41KF2b^T. Values of 39.75% ±0.35, 69.40 ±2, 49.65% ±0.50 and 61.90 were respectively obtained (Table 4). Moreover, further analysis comprising DNA-DNA relatedness of *B. xiamenensis* should also be performed, although metabolic profile and phylogenetic affiliations derived from *gyrB* and *rpoB* genes clearly distinguish these species. According the DNA-DNA criteria for bacterial species definition recommended by the *ad hoc* committee (Wayne *et al*., 1987) a similarity-threshold value of 70% must be considered. Nevertheless, some reports ambiguities were recognized (Adékambi *et al*., 2008; Nhung *et al*., 2007) when a DDH cut-off equal to or higher than 70% was applied as a threshold for delineation of species, demonstrating that this value should not be used as a strict species boundary. In fact the novel species demonstrated approximately 70% DDH similarity with type strain of *B. pumilus* ATCC 7061^T, but only ~40% with a reference strain *B. pumilus* ATCC 14884, despite the similarity on 16S rRNA, *gyrB* and *rpoB* sequence genes and phenotypic characteristics among these two *B. pumilus* collection strains, highlighting the ambiguities in the taxonomic grouping based on a rigorously DDH cut-off.

Data from the polyphasic studies described above, clearly supporting the claim that the three isolates from health´s products represented a novel species within the genus *Bacillus*, for which the name *Bacillus invictus* sp. nov. is proposed.

Our results also demonstrated: (i) the suitability of the *gyrB* and *rpoB* as adequate markers for the phylogenetic discrimination between *Bacillus invictus* sp. nov., *B. pumilus*, *B. safensis*, *B. altitudinis* and *B. xiamenensis* and (ii) the reliability and the interest of two different spectroscopic techniques (MALDI-TOF/MS and FTIR-ATR) on the differentiation of closely related *B. pumilus* group species, namely among *B. pumilus*, *B. safensis* and *B. invictus* sp. nov.

Description of *Bacillus invictus* sp. nov.

Bacillus invictus (in.vi´.ctus. adj. masc. invictus pertaining to Oporto city).

Vegetative cells are rod-shaped and 0.5 – 0.7 mm in diameter an 1.0 – 1.5 mm in length, Gram-positive, motile and catalase- and oxidase-positive. Does not grow anaerobically. Growth occurs at 25-50 ºC (optimum, 30-37 ºC) but not at 4 or 60 ºC. Growth occurs in the range 0-10% (w/v) NaCl and at pH range 6.0–8.0. Colonies are round, smooth, yellowish in color, have irregular margins and approximately 2.8 ±0.2 mm on TSA plates incubated at 37 ºC for 24 h. Nitrate is not reduced to nitrogen gas but gelatine, esculin and citrate is utilized.

ONPG, L-proline, L-leucine, L-phenylalanine are decomposed but not L-valine, L-tryptophan, L-arginine L-ornithine, L-lysine or urea. β-haemolytic on 5% sheep blood agar. Acid is produced from glycerol, D-ribose, glucose, fructose, mannose, arbutin, esculin, salicin, cellobiose, sucrose, trehalose, gentiobiose, D-tagatose and L-arabinose, but not from L-xylose, adonitol, sorbose, rhamnose, dulcitol, inositol, mannitol, D-sorbitol, methyl *α*-D-glucoside, maltose, melibiose, inulin, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, erythritol, methyl *β*-D-xyloside, melezitose, gluconate, 2-ketogluconate or 5-ketogluconate, D-arabinose and lactose. Reactions from D-xylose, D-galactose, α methyl-D-mannoside, N-acetylglucosamine and amygdalin vary among strains. The chain composition of the whole-cell fatty acids is $C_{15:0}$ iso (41.3%), $C_{15:0}$ anteiso (35.3%), $C_{17:0}$ iso (4.9%) and $C_{17:0}$ anteiso (6.6%). Quinone system is menaquinone MK-7. Polar lipid profile consist of the major lipids diphosphatIdylglycerol, an unidentified phospholipid phosphatidylglycerol two unidentified glycolipids and three lipids not containing a sugar, phosphate or amino residue. The DNA G+C content is 41 mol%. MALDI-TOF mass spectra reveal peaks with m/z values of 3690, 3824, 4305, 5272, 5950, 6100, 6704 and 7416 for $Bi._{FFUP1}$. A characteristic carbohydrate pattern by FTIR-ATR spectroscopy is also demonstrated. The type strain Bi_{FFUP1} (DSM 26896 and CCUG 64113), was isolated from health´s contaminated products in Portugal.

Table 1. Differential metabolic profiles of strain Bi.$_{FFUP1}^{T}$ and closely related *B. pumilus* ATCC 7061^{T}, *B. safensis* FO-036b^{T}, *B. xiamenensis* HYC-10^{T} and *B. altitudinis* 41KF2b^{T}.

	Bi.$_{FFUP1}^{T}$	***B. pumilus* ATCC 7061^{T}**	***B. safensis* FO-036b^{T}**	***B. xiamenensis* HYC-10^{T}**	***B. altitudinis* 41KF2b^{T}**
Carbohydrates acid production profile					
Cellobiose	+	-	+	+	+
D-Arabinose	-	+	+	-	+
Erythritol	-	+	+	-	-
Glucose	+	-	+	-	-
Inositol	-	+	-	-	-
Maltose	-	+	-	-	+
Mannitol	-	+	+	+	+
Mannose	+	+	-	+	+
Raffinose	-	+	-	+	-
Rhamnose	-	-	-	+	+
Sorbitol	-	-	-	+	+
Starch	-	-	-	+	+
Amino acid utilization					
L-Arginine	-	-	-	-	+

Table 2. *Bacillus* spp. used in this study, their sources and correspondent GenBank accession numbers.

Origin	Isolate	Year/Origin/ Location	GenBank acession numbers		
			16S rRNA gene	***rpoB* gene**	***gyrB* gene**
Health Products (Medicines)	Bi.$_{FFUP1}$ [a]	2005/health products/Pt [a]	JX183147	JX183163	JX183192
	Bi.$_{FFUP2}$ [a]	2005/health products/Pt [a]	JN699028	JX183165	JX183194
	Bi.$_{FFUP3}$ [a]	2005/health products/Pt [a]	JN699026	JX183168	JX183197
ATCC (*B. pumilus* reference strain)	ATCC14884		JF749284	KC895450	KC895462
ATCC (*B. pumilus* type strain)	ATCC 7061^{T}		GQ911554.1	EU138862.1	AY167869.1
***B. safensis* type strain**	FO-036b^{T}	1999/Clean-room air particulate/USA	AF234854	KC895451	AY167867
***B. xiamenensis* type strain**	HYC-10^{T}	2014/mullet-*Mugil cephalus*/China	JX680066	JX679987	JX680142
***B. altitudinis* type strain**	41KF2b^{T}	2006/ High-elevation air sample/India	AJ831842	JX680064	JX680219

[a] Isolates obtained to this study from Quality Control Department (INFARMED), Lisbon, Portugal.

ATCC, American Type Culture Collection, Manassas, VA, USA.

Table 3. Major fatty acids profiles of novel isolates from *Bacillus* sp. and their closely related species.

	Fatty Acids			
	iso-$C_{15:0}$	**iso-$C_{17:0}$**	**anteiso-$C_{15:0}$**	**anteiso-$C_{17:0}$**
$Bi._{FFUP1}^{T}$	41.3	4.9	35.3	6.6
B. xiamenensis HYC-10^{T}	39.1	13.1	22.7	5.8
B. altitudinis 41KF2b^{T}	46.1	7.4	25.5	5.7
B. safensis FO-036b^{T}	44.2	10.1	23.1	6.7
B. pumilus ATCC 7061^{T}	22.2	7.8	36.3	10.5

Table 4. DNA-DNA hybridization relatedness (%±SD) of the *Bacillus invictus* sp. nov. with reference strains *B. pumilus* ATCC 14884, *B. pumilus* ATCC 7061^{T}, *B. safensis* FO-036b^{T} and *B. altitudinis* 41KF2b^{T}.

Strains	*B. invictus* Bi.$_{FFUP1}$
***B. pumilus* ATCC 14884**	39.75% ±0.35
***B. pumilus* ATCC 7061^{T}**	69.40% ± 2.00
***B. safensis* FO-036b^{T}**	49.65% ±0.50
***B. altitudinis* 41KF2b^{T}**	61.90%

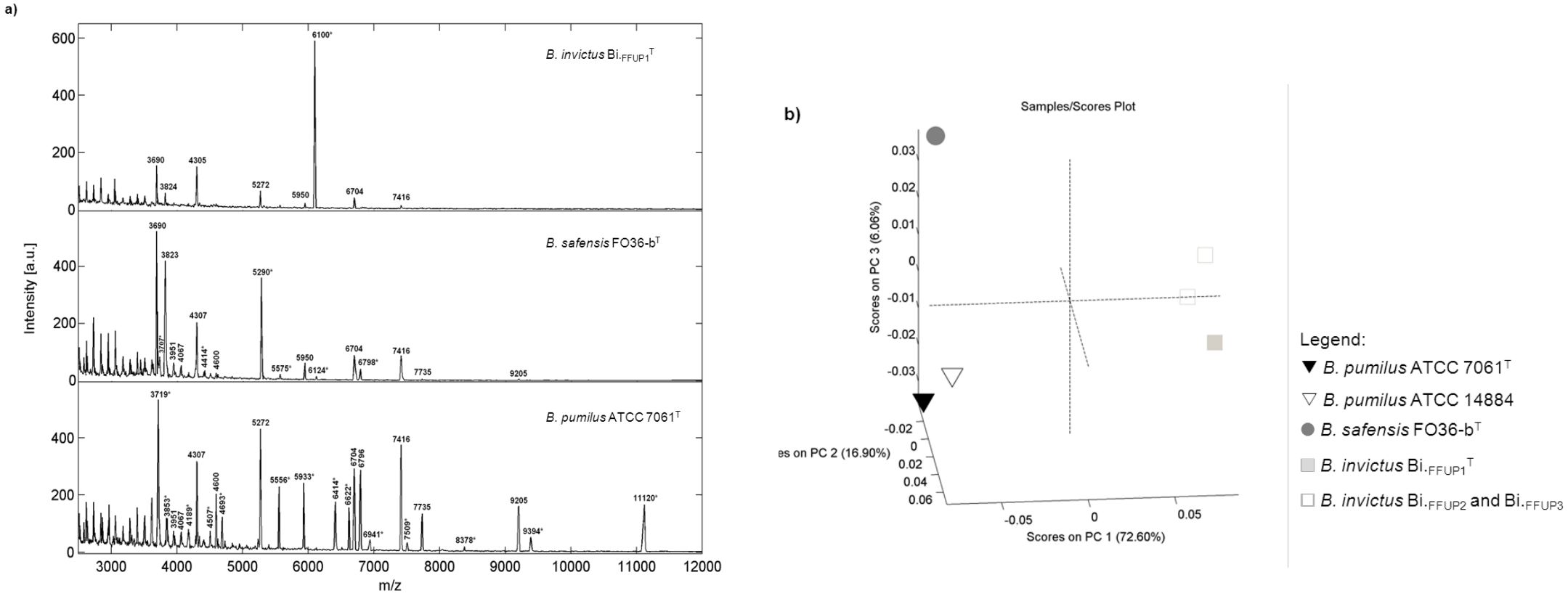

Figure 1. a) Mass spectra comparison of Bi.$_{FFUP1}$^{T} and closed related *B. safensis* FO-36b^{T} and *B. pumilus* ATCC 7061^{T} generated by MALDI-TOF/MS. Species-specific peaks are indicated by (*). A. u. means arbitrary intensity. b) Score plot corresponding to the first three components of the PCA regression model of isolates Bi.$_{FFUP1}$^{T}, Bi.$_{FFUP2}$, Bi.$_{FFUP3}$, *B. safensis* FO-36b^{T}, *B. pumilus* ATCC 7061^{T} and *B. pumilus* ATCC 14884 analyzed.

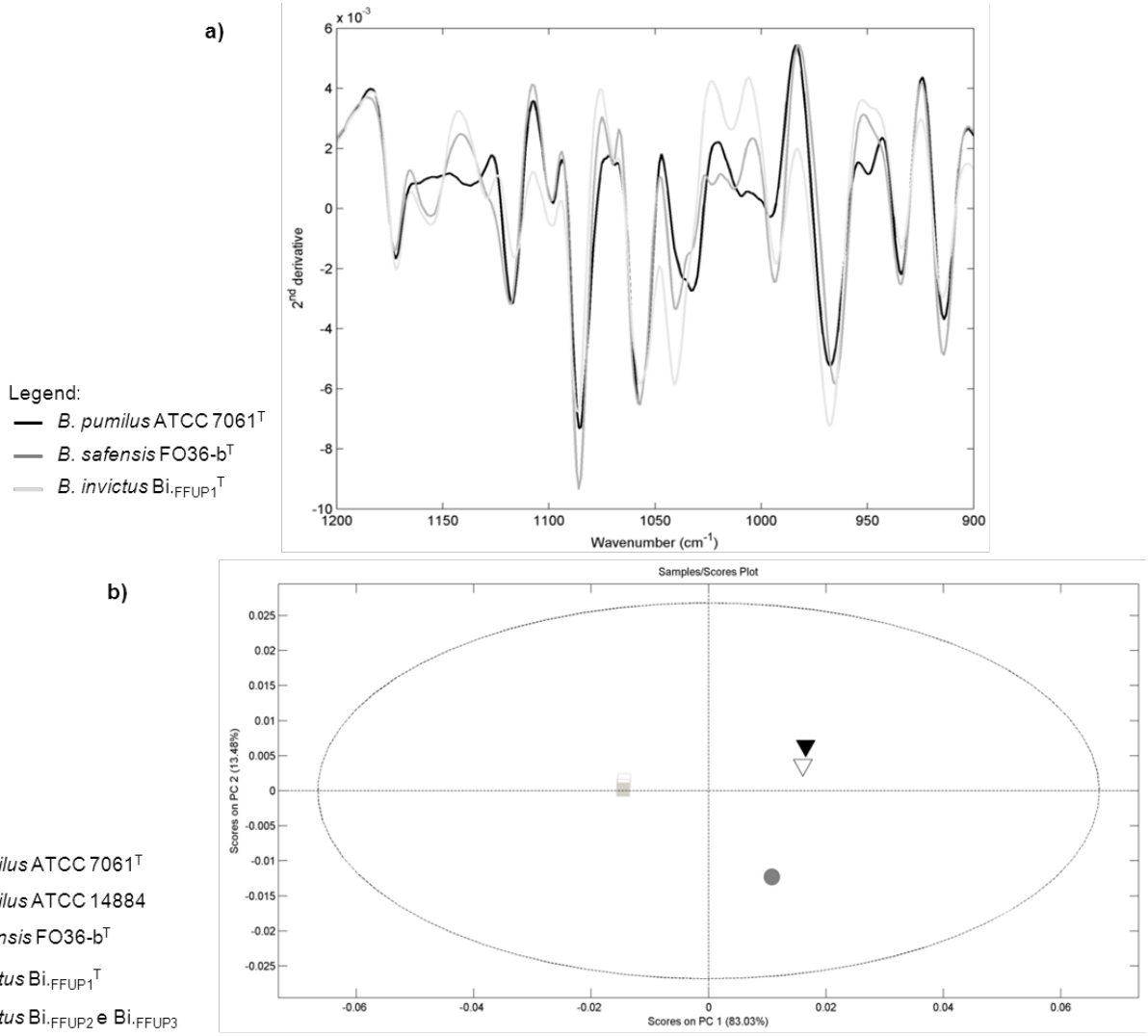

Figure 2. a) FTIR-ATR spectra of *Bacillus invictus* sp. nov. Bi.$_{FFUP1}^{T}$ and closely related *B. safensis* FO-36b^{T} and *B. pumilus* ATCC 7061^{T} processed with SNV and Savitzky-Golay (9 points filter size, 2nd degree polynomial, 2nd derivative) in the region 1200-900 cm^{-1}. b) Score plot corresponding to the first three components of the PCA regression model of isolates Bi.$_{FFUP1}^{T}$, Bi.$_{FFUP2}$, Bi.$_{FFUP3}$, *B. safensis* FO-36b^{T}, *B. pumilus* ATCC 7061^{T} and *B. pumilus* ATCC 14884 analyzed.

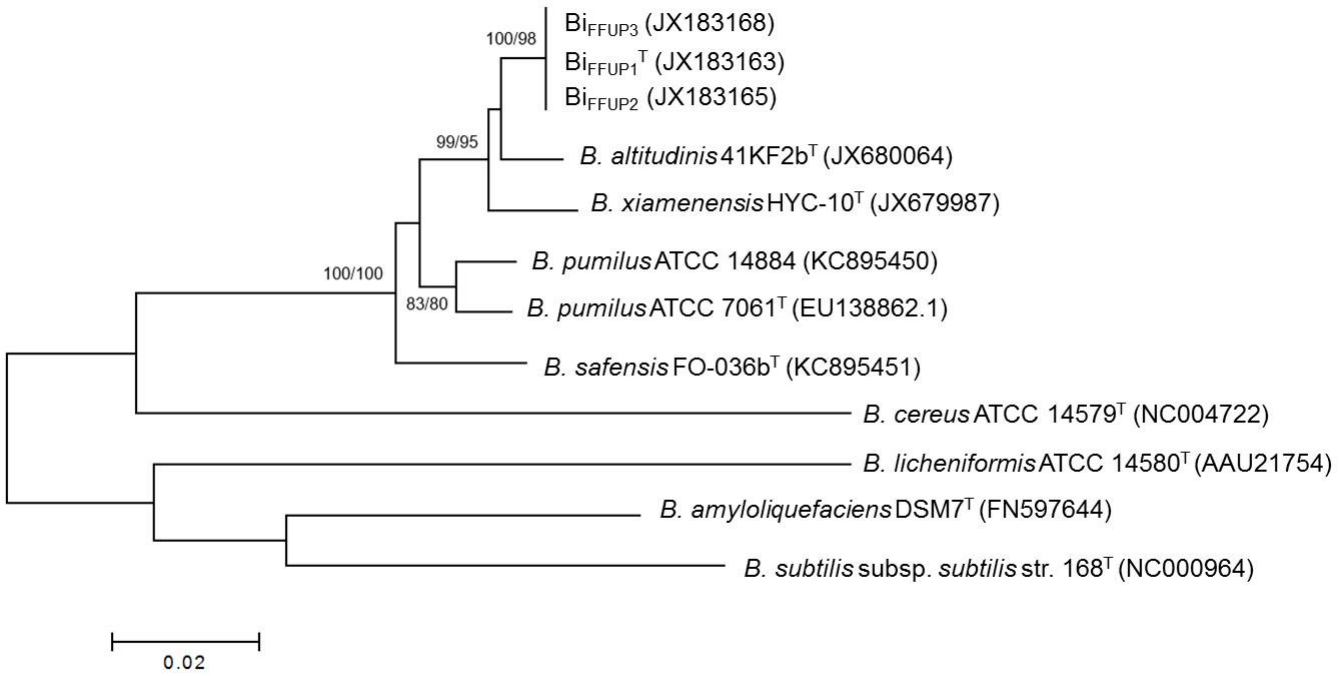

Figure 3. Neighbour-joining (NJ) tree based on the *rpoB* gene sequences comparison, showing the relationship of strains Bi.$_{FFUP1}$, Bi.$_{FFUP2}$ and Bi.$_{FFUP3}$ with related species. Genetic distances were constructed using Jukes-Cantor model. Numbers at branch points indicated bootstrap percentages from both NJ (before the slash '/') and ML analyses (after the slash '/'). As NJ tree was very similar to the ML tree, only the first is shown. *B. subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* DSM7^{T}, *B. licheniformis* ATCC 14580^{T} and *B. cereus* ATCC 14579^{T} were used as outgroups. Accession numbers were given in parentheses. Bar: genetic distance of 0.02.

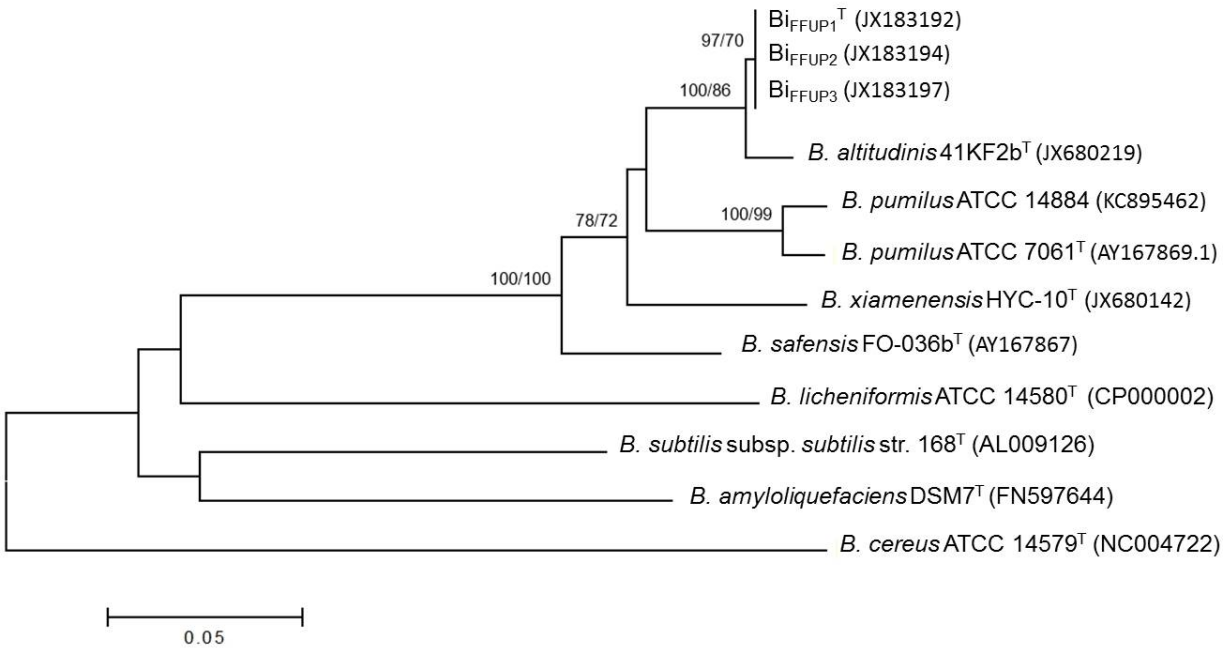

Figure 4. Neighbour-joining (NJ) tree based on the *gyrB* gene sequences comparison, showing the relationship of strains Bi.$_{FFUP1}$, Bi.$_{FFUP2}$ and Bi.$_{FFUP3}$ with related species. Genetic distances were constructed using Jukes-Cantor model. Numbers at branch points indicated bootstrap percentages from both NJ (before the slash '/') and ML analyses (after the slash '/'). As NJ tree was very similar to the ML tree, only the first is shown. *B. subtilis* subsp. *subtilis* str. 168, *B. amyloliquefaciens* DSM7^{T}, *B. licheniformis* ATCC 14580^{T} and *B. cereus* ATCC 14579^{T} were used as outgroups. Accession numbers were given in parentheses. Bar: genetic distance of 0.05.

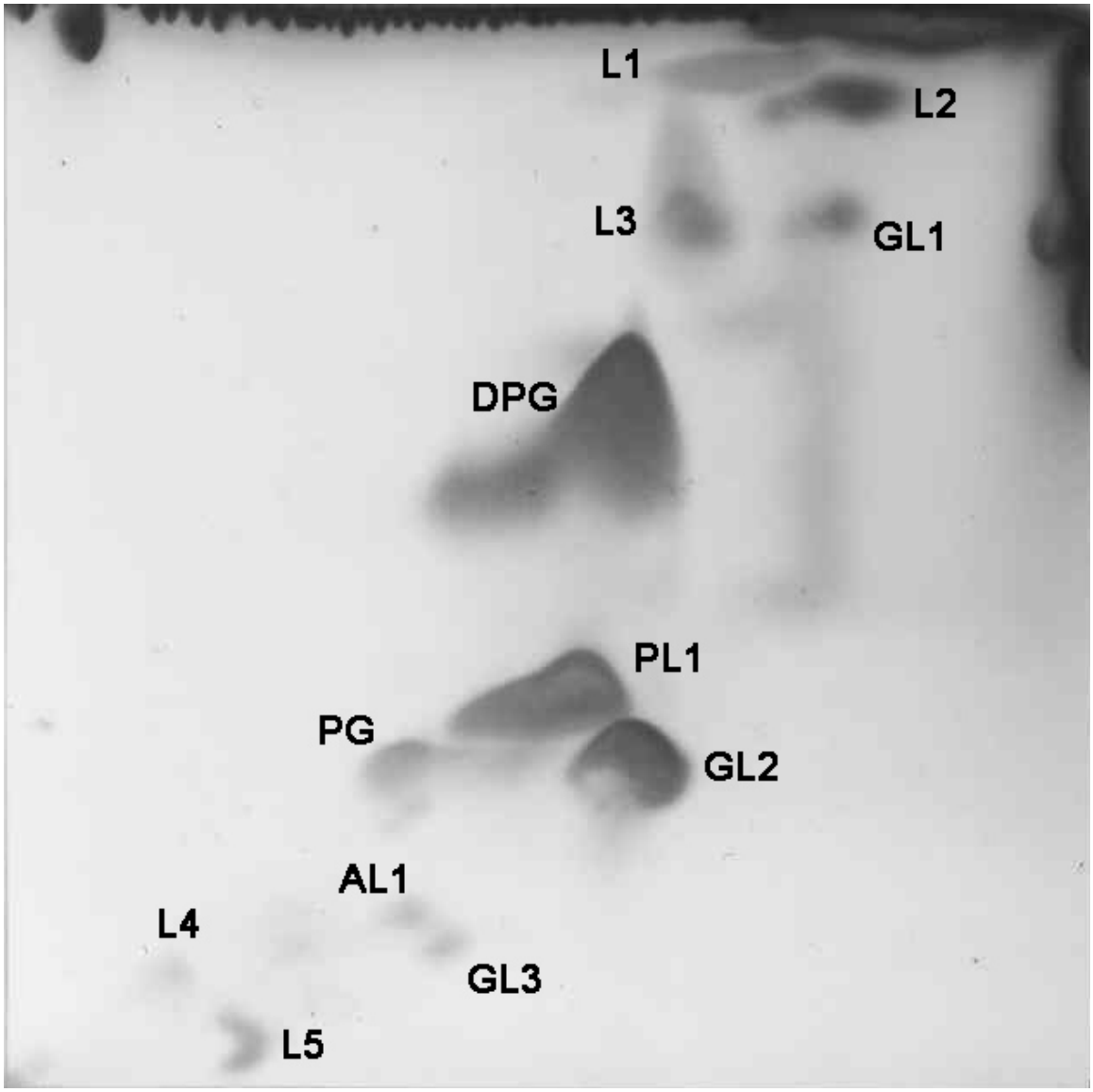

Figure 5. Total polar lipid profile of Bi.$_{FFUP1}^{T}$ after two-dimensional thin layer chromatography and detection with 5% ethanolic molybdatophosphoric acid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL1-2, unidentified glycolipids; PL1, unidentified phospholipid; AL1, unidentified aminolipid; L1-5 unidentified polar lipids not containing a phosphate, an amino or sugar residues.

Acknowledgments

Dr. Parag Vaishampayan and Dr. Kasthuri Venkateswaran are gratefully acknowledged for providing isolate *B. safensis* FO-36b. This work were funded by a PhD fellowship (Ref. SFRH / BD / 61410 / 2009) granted to Raquel Branquinho and by a post-doctoral fellowship (Ref. SFRH / BPD / 70548 / 2010) granted to Clara Sousa and PEst-C/EQB/LA0006/2011 both from FCT (Fundação para a Ciência e Tecnologia, Portugal).

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Supporting Information

Bacillus invictus sp. nov., isolated from healthy products in Portugal

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Running title: *Bacillus invictus* sp. nov.

Table S1. Physiological and phenotypic characteristics of *B. invictus* strains Bi.$_{FFUP1}^{T}$, Bi.$_{FFUP2}$ and Bi.$_{FFUP3}$ (data from this study).

Characteristics	Bi.$_{FFUP1}^{T}$	Bi.$_{FFUP2}$	Bi.$_{FFUP3}$
Growth ability			
NaCl range (%, w/v)	0-10	0-10	0-10
pH range	6-8	6-8	6-8
Temperature range	25-50 ºC	25-50 ºC	25-50 ºC
Optimum temperature	30-37 ºC	30-37 ºC	30-37 ºC
Hydrolysis of			
Esculin	+	+	+
Gelatin	+	+	+
Citrate	+	+	+
Nitrate	-	-	-
Carbohydrates acid production profile			
D-Arabinose	-	-	-
D-Galactose	+/-	+/-	+/-
L-Arbinose	+	+	+
Erythritol	-	-	-
D-Xylose	+/-	+/-	+/-
L-Xylose	-	-	-
Adonitol	-	-	-
ß-Methyl – D-Xyloside	-	-	-
Glucose	+	+	+
Fructose	+	+	+
Sorbose	-	-	-
Rhamnose	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
a-Methyl-D-Mannoside	+/-	+/-	+/-
a-Methyl-D-Glucoside	-	-	-
N-Acetyl Glucosamine	+/-	+/-	+/-
Amygdalin	+/-	+/-	+/-
Arbutin	+	+	+
Trehalose	+	+	+
Inulin	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentiobiose	+	+	+
Turanose	-	-	-
D-Lyxose	-	-	-
D-Tagatose	+	+	+
D-Fucose	-	-	-
L-Fucose	-	-	-
D-Arabitol	-	-	-
L-Arabitol	-	-	-
Gluconate	-	-	-
2-Keto Gluconate	-	-	-
5-Keto Gluconate	-	-	-
Cellobiose	+	+	+
Lactose	-	-	-
Maltose	-	-	-

Mannose	+	+	+
Melezitose	-	-	-
Melibiose	-	-	-
Raffinose	-	-	-
D-Ribose	+	+	+
Salicin	+	+	+
Starch	-	-	-
Sucrose	+	+	+
Dulcitol	-	-	-
Glycerol	+	+	+
Mannitol	-	-	-
Amino acid utilization			
L-Arginine	-	-	-
L-Leucine	+	+	+
L-Proline	+	+	+
L-Ornithine	-	-	-
L- Phenylalanine	+	+	+
L-Tryptophan	-	-	-
L-Lysine	-	-	-
L-Valine	-	-	-

Table S2. PCR primers sequences and amplification conditions for detection of 16S rDNA, *rpoB* and *gyrB* genes.

Gene	Primer name	Primer sequences 5´- 3´	PCR amplification				References
			Denaturation	Annealing	Cycles	Final extension	
16S rDNA	Seq_A	AGAGTTTGATCHTGGYTYAGA	94ºC for 30 s	55ºC for 60 s	30	72ºC for 120s	[6]
	Seq_B	ACGYTACCTTGTTACGACTTC					
β-subunit of RNA polymerase (*rpoB* gene)	$rpoB_{1\_fw}$	CAGAAGCTACGCACGCATAA	94ºC for 30 s	56ºC for 60 s	30	72ºC for 120s	This study
	$rpoB_{1\_rv}$	GCGTCCAACATTTGCTAGGT					
	$rpob_{2\_fw}$	CAACACGCTGGAAAAAGACA					
	$rpoB_{2\_rv}$	TTCTCGGCGTCACTTTACCT					
	$rpoB_{3\_fw}$	CACCAGAGGGTCCAAACATT					
	$rpoB_{3\_rv}$	GGTTGCGTAAAGCATCTTCC					
	$rpoB_{4\_fw}$	CATGAGTGAGCGCCTTGTAA					
	$rpoB_{4\_rv}$	CGTCTGCTTTCTTCGTTTCC					
β-subunit of DNA gyrase (*gyrB*)	UP-1S	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	94ºC for 60 s	60ºC for 60 s	30	72ºC for 120s	[23]
	UP-2Sr	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT					

2.3. Differentiation of *Bacillus pumilus* and *Bacillus safensis* using MALDI-TOF/MS

Publications:

Differentiation of *Bacillus pumilus* and *Bacillus safensis* using MALDI-TOF/MS

In this study, supported on a diversity of isolates and using vegetative cell forms treated with a protocol enriching proteins, were demonstrated the ability of MALDI-TOF/MS to provide a reproducible and significant protein profiling of *B. pumilus* and *B. safensis*. Moreover, for the first time, a combination of MALDI-TOF/MS and chemometric methods were proved to differentiate these closed species. Species-specific peaks were identified, being also for the first time some of them putatively assigned as ribosomal or spore's proteins.

Differentiation of *Bacillus pumilus* and *Bacillus safensis* using MALDI-TOF/MS

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Running title: *B. pumilus* and *B. safensis* profiling by MALDI

Keywords: microorganism identification; MALDI-MS; *Bacillus pumilus*; *Bacillus safensis*; chemometrics

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) is increasingly used as a rapid method for microbial identification at different taxonomic levels. This method is of especially interest in species difficult to differentiate by other conventional methodologies, such as *Bacillus pumillus* and *Bacillus safensis*, for which biotechnological and pharmaceutical significance has been underlined by the production of several compounds with a wide range of applications.

In this study, we demonstrate the suitability of MALDI-TOF/MS protein profiling for a reliable *B. pumillus* and *B. safensis* differentiation, using a comprehensive and genotipically well characterized collection of isolates recovered from different origins and geographic locations. The resulting mass fingerprints, obtained from vegetative cell forms coupled with a protein enrichment treatment, were analyzed and characteristic peaks at the species level were assigned. Results showed that spectral fingerprints provided reproducible species-specific ion peaks signals. Using a chemometric approach the score plot generated by partial least square discriminant analysis (PLSDA) of mass spectra demonstrated the presence of two individualized clusters, each one enclosing isolates belonging to a species-specific spectral group. In addition, the generated pool of species-specific proteins was compared and some characteristic species-specific ion peaks tentatively assigned. Therefore, in *B. pumilus* the specific ion *m/z* peak of 5271 can be associated with one small acid-soluble spore protein (SASP O) or with 50S protein L35, whereas in *B. safensis* specific ion *m/z* peaks of 5288 and 5568 can be correlated with SASP J and P, respectively, and *m/z* of 6413 with 50S protein L32.

Introduction

Bacillus pumilus and *Bacillus safensis* are the most widespread and relevant terrestrial species among *Bacillus pumilus* group [1, 2, 3, 4, 5]. Their biotechnological and pharmaceutical significance has been highlighted by a wide range of applications, mostly supported on the production of peptides and/or lipopeptides with antibacterial and antifungal properties [6, 7], including as probiotics [8, 9, 10], animal feed supplements [11, 12], as well as phytosanitary-based products [1, 3] in different plants production. Moreover, these species are common contaminant agents into industrial units, namely in food and/or pharmaceutical sectors, posing a serious problem to the quality control of these areas [2, 4].

B. pumilus and *B. safensis* are difficult to distinguish on the basis of phenotypic and biochemical characteristics and present a remarkably high level of 16S rRNA gene similarity (>99%) [5]. Sequencing of housekeeping genes such as *gyrB* and *rpoB* could be used as a valuable tool to discriminate them, although difficult to implement in routine microbiology laboratories [4, 5].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS), being an accurate, fast and affordable emerging technique has been increasingly used for identification of bacteria at different taxonomical levels [13, 14]. As a proteomics technique, MALDI-TOF/MS relies on the reproducible detection of microbial protein patterns, which can be used for microbial identification by comparing experimental mass spectra with a library of known reference strains or by comparing information of species-specific biomarkers identities [15, 16].

Dickinson et al. [17], using directly spore forms of *B. pumilus* and *B. safensis* recovered from a spacecraft unit and assembly-facility surfaces proposed their differentiation based on a single *m/z* peak by MALDI-TOF/MS. Nevertheless, there is a need for further studies in order to assess the validity of this discriminatory tool. Moreover, Lash et al. [18, 19] suggested that bacterial vegetative cell forms through an adequate sample preparation encompassing protein enrichment could provide a more informative mass spectra pattern than that obtained with bacterial spore forms [18, 19].

The snapshot of different protein compositions detected by MALDI-TOF/MS may also constitute a valuable approach for the exploration of bacterial species-specific biomarkers.

Indeed, in *Bacillus* spp. ribosomal proteins and small acid-soluble spore proteins (SASPs) have been associated to several MS signals detected [19], and thus, can be used as potential species-specific biomarkers. Despite this characterization has been successfully applied to *Bacillus cereus* group species [19] it has not been considered in members of *B. pumilus* group. In this work, through the implementation of a protocol that enhances proteins extraction [19], combined with chemometric tools, we assessed the potential of MALDI-TOF/MS fingerprinting to discriminate *B. safensis* and *B. pumilus*, using a comprehensive collection of isolates. Moreover, the presumptive assignment of their species-specific protein biomarkers was also performed.

Material and Methods

Isolates collection and identification

Five *B. pumilus* isolates and twenty-two *B. safensis* previously identified by phenotypic and genotypic (16S rRNA, *gyrB and rpoB* gene sequences) methods were studied [5, 20]. They comprise diverse PFGE-types and were recovered from different geographic terrestrial locations and sources, including food samples (Norway, Italy and Africa) (n=4), plants (USA) (n=2), gastropods (Portugal) (n=3), medicines (n=6) and cosmetic (n=4) products (Portugal), clean room environments from Mars Odyssey (USA) (n=5) as well as type and reference strains (*B. pumilus* ATCC 7061^T and 14884, and *B. safensis* FO-36b^T) (table 1).

Sample preparation

For MALDI-TOF/MS analysis, soluble proteins were extracted from *Bacillus* spp. cultures grown under aerobic conditions on LB agar (Merck, Darmstadt, Germany) for 24 h at 37 °C. Bacterial cells were harvested by transferring three full loops (ca. 30 µl) from each agar plate into 20 µl of sterile water and afterward resuspended by vortexing. Sample inactivation was carried out applying the modified trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO) inactivation protocol [18], with some modifications previously established to improve the accuracy of the mass spectra [18]. Briefly, 80 µl of pure TFA was added to 20 µl of the bacterial water suspensions. After gentle shaking for 5 min at room temperature, the solution was centrifuged during 20 min at 13000 rpm at 4 ºC followed by 10-fold dilution with HPLC grade water. The supernatant was then filtered through 0.22 µm pore size filter (Millipore Corp., Bedford, MA) and stored at -20 ºC to further analyses.

Mass spectrometry methods

Snapshots of different protein compositions were detected and acquired by a MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, AB SCIEX, Framingham, MA) operating in linear positive mode. Each spectrum was the accumulated

sum of at least 2000 laser shots for *m/z* 2500-12000 due to the good reproducibility of the spectral profile in that mass range. All the spectra were externally calibrated using a commercial mixture of angiotensin I, ACTH and insulin (AB SCIEX, Framingham, MA) and analyzed with the Data Explorer software (Version 4.6, AB SCIEX, Framingham, MA).

For MALDI-TOF/MS experiments, 2 µl of the filtrated microbial dilution were mixed with 2 µl of a 12-mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich, St. Louis, MO) solution in 100% ACN and 0.3% TFA. 1 µl of the mixture was spotted onto a stainless steel MALDI sample plate and allowed to dry at room temperature. For each isolate, two biological replicates (obtained from two different agar plates) were carried out and the mean spectra were considered for the analysis. Mass spectra were baseline corrected, noise-filtered and *m/z* values were extracted from the raw experimental mass spectra that included all the mass peaks with a relative signal to noise (S/N) ratio intensity above 2.

Chemometric methods

MALDI-TOF/MS spectra were mean-centred and analysed by partial least squares discriminant analysis (PLSDA) [21]. The PLSDA scores were used as the source for hierarchical cluster analysis (HCA). The purpose of HCA was the generation of dendrograms highlighting the association between isolates. Dendrograms were performed directly on unprocessed PLSDA scores using the Euclidean distance and the Median's algorithm [22]. All chemometric models were performed in Matlab version 6.5 release 13 (MathWorks, Natick, MA) and the PLS Toolbox version 3.5 for Matlab (Eigenvector Research, Manson, WA).

Biomarker identification

Intact protein masses were used to generate a pool of candidate's proteins for the identification of specific markers. The selected distinct mass information was submitted to a web-based TagIdent software tool (http://web.expasy.org/tagident/) using 1% mass error and searching *Bacillus pumilus* as the taxonomy search tool. No restrictions on protein isoelectric point were used. This tool allowed the identification of proteins based on the experimental masses acquired by mass spectrometry using the information available at the UniProtKB/Swiss-Prot and UniProtKB/TrEMBL protein sequence databases. Moreover, ribosomal proteins of genome-sequenced type strains, including *B. pumilus*

ATCC 7061^T available in the database developed by Hotta et al. [23] were also included for comparison. For tentative assignments, N-terminal methionine loss was first considered based on the “N-end rule” [24], where N-terminal methionine is cleaved from specific penultimate amino acid residues such as glycine, alanine, serine, proline, valine, threonine and cysteine. To validate the candidate peak assignments of the species-specific-peaks, molecular masses of the presumed assignments were further confirmed by MALDI-TOF/MS in the reflector positive acquisition mode.

Results and Discussion

One of the current challenges in bacterial taxonomy is to integrate timely and accurate typing methods for a meaningful identification of microorganisms [13], which is particularly problematic for closely related species such as *B. pumilus* and *B. safensis* [2, 5]. Discrimination of these species by sequencing of molecular markers (e.g. *gyrB*), a time demanding and expensive methodology not readily available for routine laboratories, was recently demonstrated [4, 5]. Because of the range of applications, from animal feed supplements to plant-growth promoters, and medical and industrial relevance of *B. pumilus* and *B. safensis* isolates, reliable, easy and rapid methodologies for their correct differentiation are needed. Despite the interest of MALDI-TOF/MS in bacterial differentiation there are no comprehensive studies assessing its potential on *B. pumilus* and *B. safensis* discrimination. Moreover, presumptive assignment of *B. pumilus* and *B. safensis* species-specific protein biomarkers was not previously attempted.

Sample preparation conditions

Identification of bacterial species by MALDI-TOF/MS, is based on their mass profile considering analytes with low mass weight (less than 20 000 Da) obtained from whole bacterial cells suspensions. Therefore, for MS applications it is imperative to define a standardized protocol including the establishment of a rigorous sample preparation and cultivation conditions (culture medium, temperature and time), which should provide an adequate number of ion mass peaks to allow identification and discrimination of closely related bacterial species. Additionally, definition of strict parameters for spectral data acquisition is also required.

The protein enrichment reported by Lash et al [18] for *B. cereus* group members, using a protocol combining a TFA treatment, centrifugation, and filtration steps was successfully applied to *B. pumilus* and *B. safensis* isolates clonally diverse and collected from different terrestrial origins [4]. Therefore, we demonstrate that this easy sample preparation approach can be applied to these species to generate reproducible mass spectra data with sufficient number of mass peaks, reinforcing the potential for the successful application of the MS technique in routine laboratories.

Mass spectrometry analysis

The figure 1 displays arithmetic means of MALDI-TOF/MS profiles from the two *Bacillus* species under study: (a) *B. pumilus* (5 spectra) and (b) *B. safensis* (22 spectra), in the range 2000 to 12000 *m/z*. In fact, MALDI-TOF/MS profile analysis revealed the presence of species-specific mass signals. Table 2 compiles the characteristic peak masses for each spectral group and the respective *m/z* peak present in both species.

As expected, similar fingerprints could be observed for isolates belonging to the same species, exhibiting several common peaks. For instance, ion peaks at *m/z* values of 2069.5, 2170, 3060, 3608.5, 5271 and 6122 are characteristic of *B. pumilus* isolates, while *m/z* values of 2063.5, 2623.5, 2730, 3049, 3396, 5288, 5568, 6094 and 6413 are related to *B. safensis*. Moreover, *B. pumilus* and *B. safensis* isolates, demonstrated common ion peaks at m/z 2287.5, 2399.5, 2511.5, 3692.5, 3821.5, 4305.5, 5948.5, 6704, 6793.5 and 7415 (table 2), which can be considered characteristic peaks for both species, corroborating the similarities among them.

Remarkably, some spectral variability was observed within isolates belonging to the same spectral group, including the presence or absence of some ion peaks beyond those listed in table 2 (data not shown). This observation is not surprisingly, since we noticed that both species comprises a clonally diverse population, in which specific peptide profile probably reflects their evolution towards an adaptation to different niches [4].

Few studies have explored the potential of MALDI-TOF/MS to correctly identify *B. pumilus* or *B. safensis* isolates [17, 25, 26, 27]. The characterization of an unknown spectrum can be limited to the bacterial species present in a specific database and therefore, bacterial identification is only possible inside the frame of this reference library. Moreover, few well characterized *B. pumilus* isolates are available in public databases, as SpectraBank (http://www.spectrabank.org), namely the type strain ATCC 7061^T and the reference strains ATCC 14884, and no *B. safensis* was yet included, which constrains its identification.

Farfour et al. [25] applying MALDI-TOF/MS supported by Andromas database and analyzing the samples without previous preparation, showed that it was not possible to differentiate *B. pumilus* and *B. safensis* isolates, highlighting the need for the improvement

and enlargement of the database and eventual sample treatment before MS analysis to improve sensitivity.

Moreover, using *B. pumilus* reference strains (ATCC 7061^T and 14884) and a sample extraction procedure comprising bacterial biomass harvest in a solvent followed by a centrifugation step, Böhme et al. [26] suggested the presence of ion peak values at *m/z* of 3620, 5297, 6617 and 7237 as specific for this species when compared with *Bacillus subtliis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus thuringiensis* and *Bacillus cereus*. In addition, the presence of *m/z* of 3620, 6617 and 7238 were also detected by Fernández-No et al. [27] in the same reference strains. Analysis of our MALDI-TOF/MS profiles did not reveal these ion *m/z* peaks as *B. pumilus* species-specific, nevertheless, a closer inspection of mass spectra of *B. pumilus* ATCC 7061^T and 14884 recognizes the presence of ion peaks at *m/z* values of 3621, 5290 and 6624, although the peak 7238 *m/z* was not detected. The differences found in these bacterial profiles can be justified by the distinct culture medium used and the sample preparation procedure employed.

Dickinson et al. [17] presented MALDI-TOF/MS as a useful taxonomic tool for differentiating spores of *B. pumilus* and *B. safensis*. Results revealed the presence of two groups of characteristic peaks, comprising *B. pumilus* (*m/z* of 6860, 7230 and 9606) and *B. safensis* (*m/z* of 6860, 7230, 7620 and 9606). The authors claimed the presence of the additional ion peak at *m/z* of 7620 Da in the spectra profile of *B. safensis* as a species-specific biomarker, allowing the discrimination of these two species. Nonetheless, analysis of our *B. safensis* spectral data (n=22) did not reveal the presence of this *m/z* peak, probably because the obtained MALDI-TOF/MS profiles were from vegetative cell forms. Additionally, the spectral profile obtained from spore cell forms seems to be insufficient to appropriately discriminate among these closely related species, since spectral data with few number of ion peaks were generated.

Chemometric analysis

B. pumilus and *B. safensis* were clearly discriminated by a combined MALDI-TOF/MS and chemometrics approach. Score plot generated by partial least square discriminant analysis (PLSDA) of mass spectra of all isolates tested, exhibited two individualized clusters, each one enclosing isolates belonging to a particular spectral group, which included reference and type strains (Fig. 2) of (a) *B. pumilus* and (b) *B. safensis*. The

PLSDA scores were also presented in a form of dendrogram corroborating the species discrimination into two distinct clusters (Fig. 2). Moreover, this approach allowed the discrimination of the two *Bacillus* species with 100% of sensitivity and specificity.

Despite the recognized proficiency of chemometric tools for the analysis and identification of bacteria based on their fingerprint, in the case of *Bacillus* spp, this characterization was only previously applied in *B. cereus* group species [19]. Therefore, this is the first successful application of this approach considering members of *B. pumilus* group, stressing its relevance for discrimination among closed related species.

Putative molecular biomarkers assignments

The possibility of biomarkers identification is one of the most valuable aspects of the MS-based identification techniques. The assignment of 25 mass signals in the spectra of *B. pumilus* and *B. safensis* represent the first consistent evidence of relation between these ion *m/z* peaks and the specific candidate protein sequences, which are presented in table 3. Nevertheless, as only two *B. pumilus* genomes, with numerous proteins defined as unknown can be found in UniProtKB/Swiss-Prot and UniProtKB/TrEMBL (*B. pumilus* SAFR-032 and ATCC 7061^{T}) and no *B. safensis* were deposited, the identification of putative biomarkers is hindered. In fact, this type of assignments can be only tentatively used to establish potential connections between protein sequences and ion mass signals, and thus, should be prudently interpreted.

Ribosomal proteins and small, acid-soluble spore proteins (SASPs) have been suggested to be responsible for many mass signals detected by MALDI-TOF/MS profiles [19]. Since up to 21% of the overall cellular protein content is ribosomal and because of the fact that ribosomal proteins are part of the cellular translational machinery constitutively expressed in vegetative cells, they constitute a stable ensemble of protein biomarkers suitable for use by fingerprinting techniques [28]. Moreover SASPs, a group of species-specific proteins present in large amounts in the core region of *Bacillus* endospores, have been also suggested as biomarkers for rapid differentiation and identification of *Bacillus* spp. using mass spectrometry approaches [29, 30, 31].

We found evidences of *B. pumilus* and *B. safensis* specific biomarkers, associated with ion peaks at *m/z* 4305.5, 5948.5, 6793.5 and 7415, which can correspond to 50S ribosomal subunits proteins, respectively, L36, L33, L28 and L35 of *B. pumilus* SAFR-032

(correspondent amino acidic sequences were also presented in table 3). Moreover, for the remaining ion peaks detected, four were not assigned (*m/z* of 2287.5, 2399.5, 2511.5 and 3821.5) and two showed correspondences with membrane proteins of *B. subtilis* subsp. *subtilis* str 168, the YyzG and YjzG at *m/z* of 3692.5 and 6704, respectively.

The tentative assignment of *B. pumilus* specific biomakers revealed the possible correspondence of the ion peak at *m/z* 5271 with the 50S ribosomal subunit protein L34 or with a SASP O and the *m/z* of 6122 with an uncharacterized membrane protein (YyzG) or with a transcriptional regulator – SlrA of *B. subtilis* subsp. *subtilis* str 168. Additionally, ion peaks at *m/z* of 2069.5, 2170, 3060 and 3608.5 were not possible to assign.

Concerning *B. safensis* specific ion peaks at *m/z* of 5288, 5568 and 6413, potentially correspond with two specific SASP (SASP J and SASP P) and with a 50S ribosomal subunit protein L32 also found in *B. pumilus* SAFR-032. The remaining ion peaks detected were not possible to designate. Therefore, the proposed characteristic biomarkers, which could be used to differentiate between *B. pumilus* and *B. safensis* are summarized in table 4.

In order to proof these putative peak assignments, molecular masses of species-specific peaks were evaluated using MALDI-TOF/MS in reflector positive mode, where the degradation of peptides was avoided to confirm its molecular weights (Fig. 3).

Thus, our results suggest that ribosomal and spore proteins constitute most of the *B. pumilus* and *B. safensis* biomarkers. A more detailed analysis could be carried out with MS/MS peptide fragmentation of the specific proteins assigned and subsequent comparison in protein databases or even with MS/MS peptide *de novo* sequencing. Nevertheless, within the context of the present work, which aimed to establish a MALDI-TOF/MS fingerprint classification for *B. pumilus* and *B. safensis* these results may be beneficial and improve further accuracy of MS-based detection methods in identifying these species.

Conclusion

MALDI-TOF/MS profiles combined with chemometric analysis (PLSDA) proved to be valuable tools for discrimination of *B. pumilus* and *B. safensis*, allowing its rapid identification. These high throughput approaches should be promptly considered for *Bacillus* species identification due to the inaccuracy of conventional techniques in the identification of closely related species of this genus. In this sense, it is imperative to standardize a sample preparation protocol, which should include a protein extraction and enrichment step, to provide informative and reproducible mass spectra. Moreover, tentative assignment of *B. pumilus* and *B. safensis* protein biomarkers suggest that most of them are ribosomal and spore proteins.

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Table 1. Origins of *Bacillus* spp. isolates (n=27) included in this study.

Isolate	Origin	Year/Location	References
Bacillus pumilus			
Bp ATCC14884			Reference strain
Bp ATCC 7061^{T}			Type Strain
Bp7		2005/Portugal[1]	
Bp11	Medicine´s (n=3)	2005/Portugal[1]	[32]
Bp15		2005/Portugal[1]	
Bacillus safensis			
Bs1		2004/Portugal[1]	
Bs2	Animals Gastropods (n=3)	2005/Portugal[1]	
Bs3		2007/Portugal[1]	
Bs13		2005/Portugal[1]	
Bs16	Medicine´s (n=3)	2005/Portugal[1]	[32]
Bs17		2005/Portugal[1]	
Bs5		2002/Portugal[1]	
Bs18	Cosmetic´s (n=4)	2002/Portugal[1]	
Bs19		2002/Portugal[1]	
Bs27		2002/Portugal[1]	
Bs24		2004/Italy[2]	
Bs25	Food´s /salame (n=3)	2004/Italy[2]	[33]
Bs33		2004/Italy[2]	
Bs22	Plant Growth Promoters (PGPR) (n=2)	1997/USA[3]	[34]
Bs23		1997/USA[3]	
Bs31	Food/beans (n=1)	2003/Africa[4]	[35]
Bs FO-36b^{T}	Clean-room/ air particulate (n=1)	1999/USA[5]	Type Strain, [21]
Bs35	Clean-room/floor (n=1)	2001/USA[5]	
Bs36	Clean-room/cabinet top (n=1)	2001/USA[5]	
Bs37	Clean-room/Mars Odyssey spacecraft surface (n=2)	2001/USA[5]	[2]
Bs38			
Bs42	Clean-room/anteroom (n=1)	2001/USA[5]	

[1]Isolates obtained from the Quality Control Department (INFARMED), Lisbon, Portugal.

[2]Isolates FEL 55 from salame felino, UNG22 from salame ungherese and MIL46 from salame milano obtained from the Istituto di Scienze delle Produzioni Alimentari (ISPA), Bari, Italy.

[3]Isolates SE 49 (AP3) and SE 52 (AP7) from cucumber roots obtained from the Culture collection of the Department of Entomology and Plant Pathology, Auburn University, Alabama, USA.

[4]Isolates Bs31 from African locust beans for ***Soumbala*** **production** obtained from Ouagadougou, Africa.

[5]Isolates F036-b, SAFN-027, SAFN-037, KL-052, 51-3C and 82-2C from spacecraft and assembly-facility surfaces obtained from California Institute of Technology, California, USA.

Table 2. Species-specific ion peaks values (average) of *B. pumilus* and *B. safensis* isolates.

	Experimental average *m/z* values*	
	B. pumilus	***B. safensis***
B. pumilus	2069.5 2170 3060 3608.5 5271 6122	
B. safensis	2287.5 2399.5 2511.5 3692.5 3821.5 4305.5 5948.5 6704 6793.5 7415	2063.5 2623.5 2730 3049 3396 5288 5568 6094 6413

* average *m/z* values (±2Da)

Table 3. Overview of biomarkers tentative assignment of MALDI-TOF/MS mass signals of *B. pumilus* group species. Protein identity was determined by the TagIdent software and compared with ribosomal subunit proteins developed by Hotta et al [23] described in Materials and Methods section.

Specie(s)	Observed Mass (Da)	Predicted Mass (Da)	UniProt Accession ID	Protein Description	Peptide sequence	Organism[c]
B. pumilus	2069.5	NA	Unassigned	NA		NA
	2170	NA	Unassigned	NA		NA
	3060	NA	Unassigned	NA		NA
	3608.5	NA	Unassigned	NA		NA
	5271	5270	A8FJG4	50S [b]RP subunit L34	MKRTFQPNNRKRSKVHGFRSRMSSKNGRLVLKRRRSKGRKKLSA	*B. pumilus* SAFR-032
		5266.89	A8FDQ9	SASP O	MTKRKANHVINGMNAAKSQGNGAGYIEDDQLVLTAEQRQNNKKRKKNQ	*B. pumilus* SAFR-032
	6122	6114	C0H3U0	Uncharacterized membrane protein YyzG	MQTNRVILLAVMICLVSAITVFLLNGCKVDFLDIGGTIIGCFLGIFVVVRIQKKQS	*B. subtilis* subsp. *subtilis* str. 168
		6126	P0C8M5	Transcriptional regulator SlrA	MKTHVKKDLDKGWHMLIQEARSIGLGIHDVRQFLESETASRKKNHKKTVRQD	*B. subtilis* subsp. *subtilis* str. 168
B. safensis	2063.5	NA	Unassigned	NA		NA
	2623.5	NA	Unassigned	NA		NA
	2730	NA	Unassigned	NA		NA
	3049	NA	Unassigned	NA		NA
	3396	NA	Unassigned	NA		NA
	5288	5299.88*	A8FHD4	SASP J	MSFFQKDKKAKSEKDHKQVDQLLEEASKELAGDPLQEAVQKKKNNDQ	*B. pumilus* SAFR-032
	5568	5544	A8FDQ8	SASP P	MTNKNTGKDIRQNSPKEHQSGQPEPLSGSKKVKNRNHTRQKHNSHHDM	*B. pumilus* SAFR-032
	6094	NA	Unassigned	NA		NA
	6413	6411.7*	A8FCW7	[b]RP subunit L32	MAVPFRRTSKMKKRLRRTHFKLQVPGMVACPECGEMKISHRVCKSCGTYKGKDVKSN	*B. pumilus* ATCC 7061[T]
B. pumilus and *B. safensis*	2287.5	NA	Unassigned	NA		NA
	2399.5	NA	Unassigned	NA		NA
	2511.5	NA	Unassigned	NA		NA
	3692.5	3698	C0H3V1	UPF0752 membrane	MSGYSNGGGYGGISSFALIVVLFILLIIVGTAFVGGF	*B. subtilis* subsp.

				protein YczN		*subtilis* str. 168
	3821.5	NA	Unassigned	NA		NA
	4305.5	4305	A8F9A9	50S [b]RP subunit L36	MKVRPSVKPICEKCKVIRRKGKVMVICENPKHKQKQG	*B. pumilus* SAFR-032
B. pumilus and *B. safensis*	5948.5	5932	A8FF72	50S [b]RP subunit L33 2	MRVNITLACTECGERNYITKKNKRNNPDRVEFKKYCSRDKKQTVHRETK	*B. pumilus* SAFR-032
	6704	6691	C0H3Z1	Uncharacterized protein YjzG	MMKNGFAYKNGKLVNIFCGKEELYNELKAFLVKTFSINVKEVSRPSIYRRTKSKQLE	*B. subtilis* subsp. *subtilis* str. 168
	6793.5	6793*	B4AE40	50S [b]RP subunit L28	MARKCVITGRKTKAGNNRSHAMNSTKRTWGANLQKVRILVDGKPKRVYVSARALKSGKVERV	*B. pumilus* ATCC 7061[T]
	7415	7410.8*	B4AM90	50S [b]RP subunit L35	MPKMKTHRGSAKRFKKTGSGKLKRSHAYTSHLFANKSTKQKRKLRKSAIVSAGDFKRIKQQLANIK	*B. pumilus* ATCC 7061[T]

[a]SASP - Small, acid-soluble spore protein.

[b]RP – Ribosomal protein.

[c]Organism – bacterial strain where the protein was described.

NA – not applicable.

* Predicted Mw proposed by Hotta et al [23], considering “N-end rule” [24] where N-terminal methionine is cleaved from specific penultimate amino acid residues such as glycine, alanine, serine, proline, valine, threonine and cysteine.

Table 4. Candidate species-specific biomarkers assignments of *B. pumilus* and *B.safensis*.

	Species	
Ion peaks (*m/z*)	***B. pumilus***	***B. safensis***
4305.5	+	+
5271	+	-
5288	-	+
5568	-	+
5948.5	+	+
6122	+	-
6413	-	+
6793.5	+	+
7415	+	+

Peak masses are presented as *m/z* values. The presence/absence of a peak in each spectral group is represented by +/-, respectively.

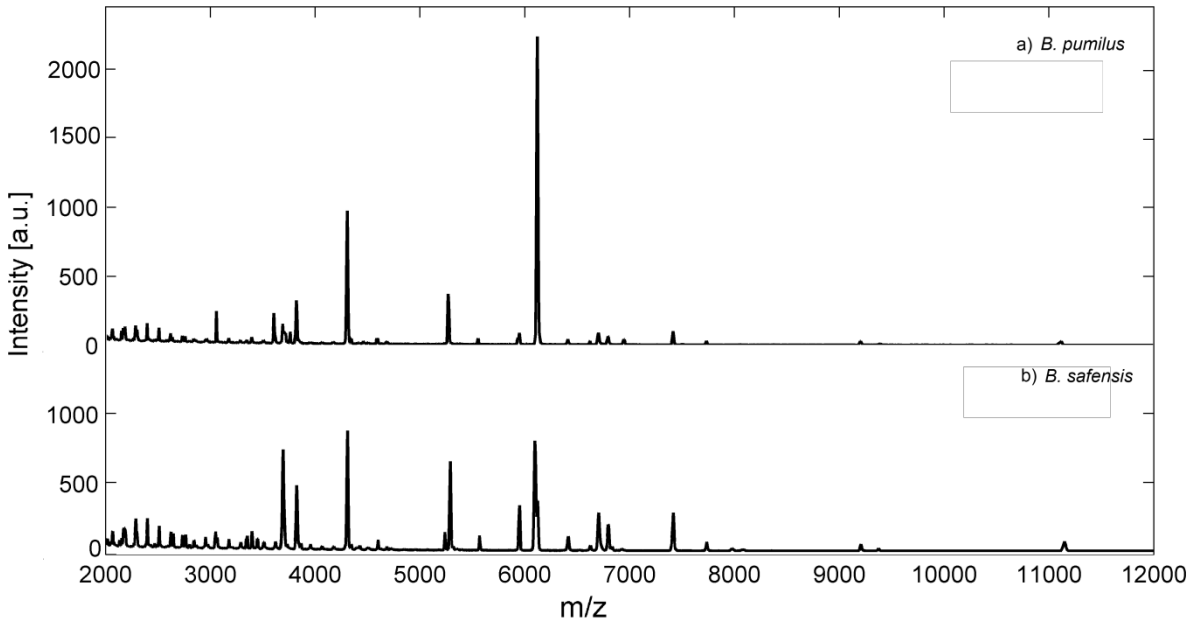

Figure 1. Mean mass spectra obtained by MALDI-TOF/MS of (a) *B. pumilus* (5 spectra), (b) *B. safensis* (22 spectra) in the range 2000 to 12000 *m/z*. Spectra were obtained by averaging the respective experimental mass spectra from all isolates (see text for details).

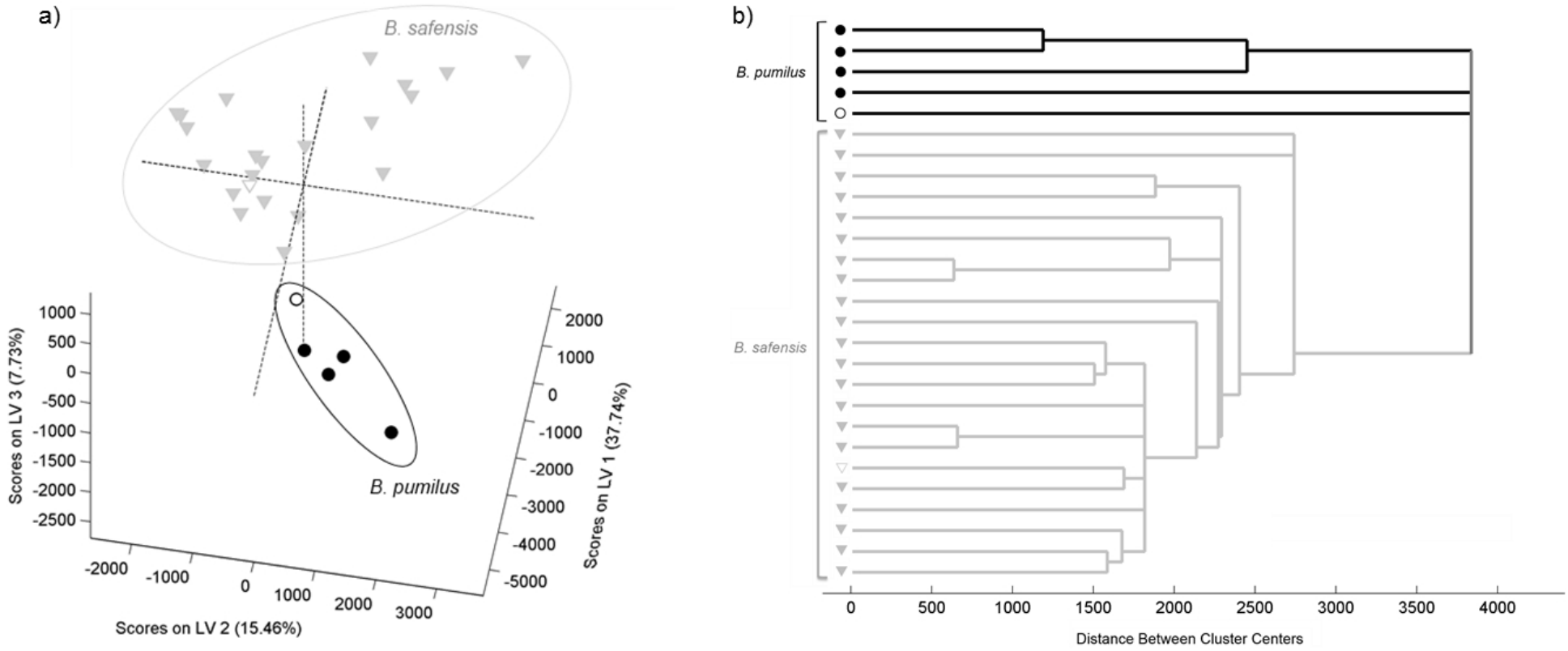

Figure 2. Score plot of the PLSDA regression model (a) and respective dendrogram (b), with *B. pumilus* and *B. safensis* isolates. Legend: ● *B. pumilus* isolates and ▼ *B. safensis* isolates. Unfiled symbols correspond to the type strains of both species.

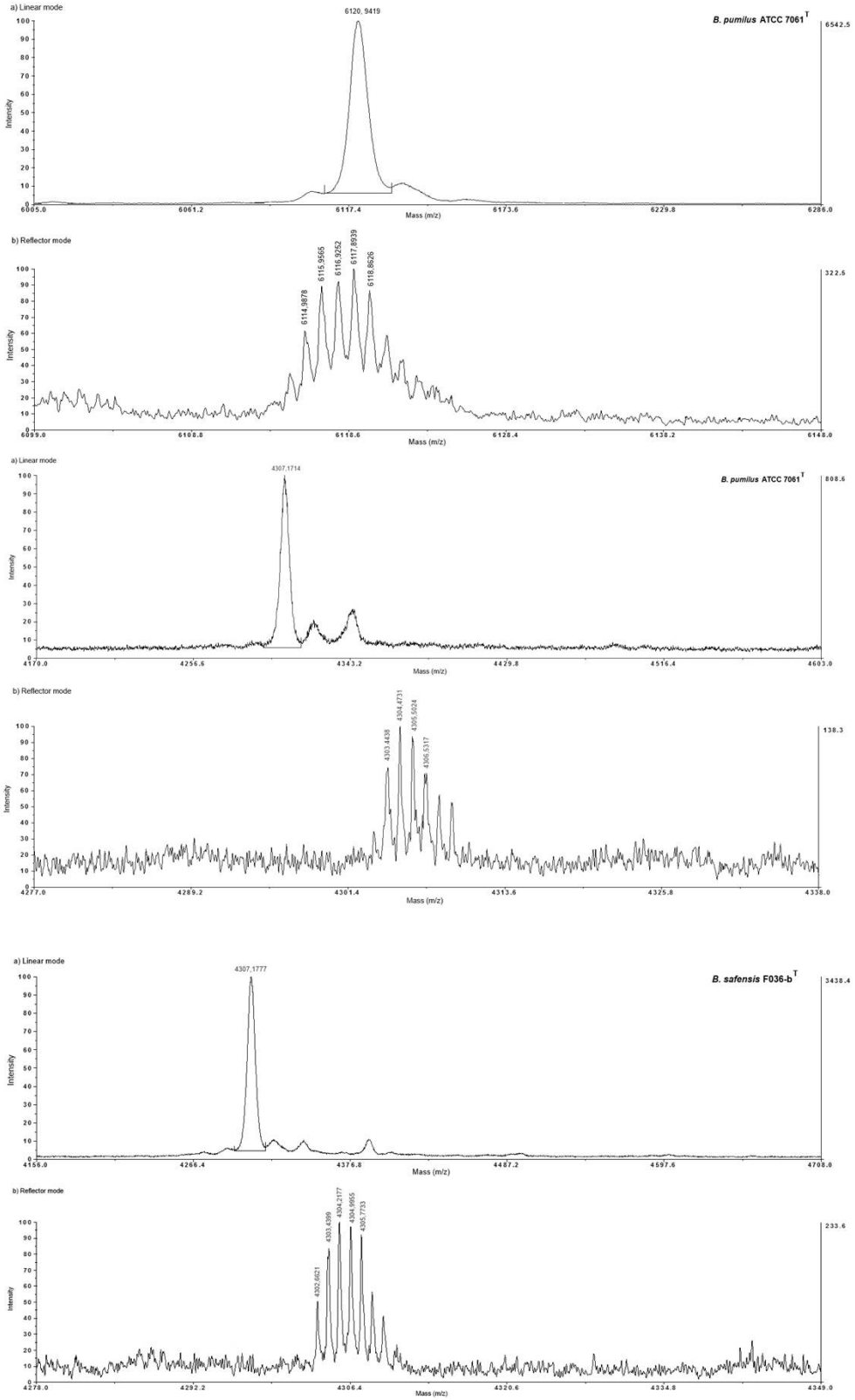

Figure 3. Representative description of the evaluation of molecular masses of species-specific peaks using MALDI-TOF/MS in linear mode (a) and the correspondent reflector positive mode (b) of *B. pumilus* ATCC 7061^T and *B. safensis* F036-b^T spectra.

2.4. Differentiation of *Bacillus pumilus* and *Bacillus safensis* by Fourier transform infrared spectroscopy and chemometry

Publications:

Differentiation of *Bacillus pumilus* and *Bacillus safensis* by Fourier transform infrared spectroscopy and chemometry

In this study was demonstrated the ability FTIR-ATR combined with chemometrics to discriminate *Bacillus pumilus* and *Bacillus safensis*. Moreover, these results are of high significance due to the current limitations in the discrimination of these two species, either by phenotypic and genotypic methods, and the rapidity and low cost of the FTIR-ATR technique.

Differentiation of *Bacillus pumilus* and *Bacillus safensis* by Fourier transform infrared spectroscopy and chemometry

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Keywords: FTIR-ATR, infrared spectroscopy, *Bacillus pumilus*, *Bacillus safensis*, chemometrics

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Abstract

In this work we assessed the ability of Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) combined with partial least squares discriminant analysis (PLSDA), to discriminate *Bacillus pumilus* and *Bacillus safensis*, two closely related species belonging to *B. pumilus* group and of high relevance for the food sector, either as they are used as probiotic in human and animals or as food contaminants. Twenty-four previously well characterized *B. pumilus* and *B. safensis* isolates recovered from different origins and geographic locations were tested. Infrared spectra of both species present a very similar peak pattern, underlining their similarity, with only few differences in the regions of carbohydrates and phospholipids/DNA/RNA vibrations. The score map obtained from the PLSDA model showed two perfectly distinct clusters, each one containing one single species. This accurate, quick and low cost vibrational spectroscopic technique proved to be able to discriminate isolates from these closely related species often difficult to identify by standard phenotypic and genotypic methods.

1. Introduction

Bacillus genus is ubiquitous in nature and some of its species as *Bacillus pumilus* and *Bacillus safensis* owns a high biotechnological and pharmaceutical interest, with a wide range of applications in the food sector, namely as animal feed supplements (EFSA, 2011), antibacterial and/or antifungal agents (Lehman et al., 2001; Aunpad et al., 2007). On the other hand, they can be found as contaminants in liquid packaging boards and blanks (Pirttijärvi et al., 1996), raw milk taken from farm milk machines and bulk tanks, milk tankers, diary silos and pasteurised milk (Sutherland & Murdoch, 1994; Coorevits et al., 2008) and gelatin (de Clerck et al., 2004) being also responsible for several foodborne illness outbreaks (From et al 2007; Van Doren et al., 2013). Moreover, under stressful conditions, they can produce highly resistant spores to physical and chemical agents that can remain in a dormant state for very long periods and undergo germination due to temperature abuse during storage and handling, which represent a serious threat in food preservation (Slepecky & Hemphill, 2006; Stecchini et al., 2013).

B. pumilus and *B. safensis* are difficult to distinguish on the basis of phenotypic and biochemical characteristics, presenting a remarkably high level of 16S rRNA gene similarity (>99%) (Satomi et al., 2006) leading to frequent erroneous identifications. Sequencing of *gyrB*, a housekeeping gene, could be used as a valuable tool to discriminate them (Liu et al, 2013), although this approach is time-consuming, laborious and expensive. Fourier transform infrared spectroscopy (FTIR) has been successfully applied over the past decades to bacterial identification of pure cultures at different taxonomic levels (Nauman 1991, Maquelin 2002, Sousa et al. 2012, Vaz et al. 2012, Sousa et al. 2014) and showed a great potential even when microorganisms are embedded in complex matrices as food (Argyri et al., 2010; Davis et al., 2010). This spectroscopic technique due to its inherent sensitivity, small sample size requirement, rapidity, simplicity and high reproducibility (Blum & Harald, 2012) became a reliable alternative to discriminate and/or identify bacterial isolates.

In this work we proved the ability of Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) combined with chemometrics, namely partial least squares discriminant analysis (PLSDA), to accurately discriminate the closely related species *B. pumilus* and *B. safensis* using a collection of isolates recovered from diverse settings and geographic origins.

2. Material and methods

2.1. Bacterial isolates

Four *B. pumilus* isolates and twenty *B. safensis* previously identified by phenotypic and genotypic (16S rRNA, *gyrB and rpoB* gene sequences) methods were studied. This collection comprises isolates of diverse PFGE-types, recovered from different geographic terrestrial locations and sources mainly from food samples (Italy and Africa) (n=4), plants (USA) (n=2), gastropods (Portugal) (n=3), health (n=6) and cosmetic (n=4) products (Portugal) and clean room environments from Mars Odyssey (USA) (n=3). The type strains *B. pumilus* ATCC 7061^T and *B. safensis* FO-36b^T were also included (table 1). Details about the collection are provided in table1.

2.2. FTIR-ATR experiments

Bacillus isolates were grown on Mueller Hinton agar (37ºC, 16h) and colonies were directly transferred from the agar plates to the ATR crystal. FTIR-ATR spectra were acquired using a PerkinElmer Spectrum BX FTIR System spectrophotometer with a PIKE Technologies Gladi ATR accessory from 4000-600 cm^{-1} with a resolution of 4 cm^{-1} and 32 scan co-additions. For each isolate, three instrumental replicates (obtained in the same day) and two biological replicates (obtained in two different days from different agar plates) were obtained and analysed corresponding to a total of six spectra for each isolate. Between each isolate measurement, a background was acquired.

2.3. Chemometric analysis

FTIR-ATR spectra were analysed by partial least squares discriminant analysis (PLSDA) after being processed with standard normal variate (SNV) (Næs et al., 2002) followed by the application of a Savitzky-Golay filter (7 smoothing points, 2nd order polynomial and first derivative) (Savitzky & Golay, 1964) and mean-centred. PLSDA is a supervised chemometric method based on the PLS regression method (Geladi & Kowalsky, 1986; Alsberg et al., 1998) and it requires a previous knowledge of assigned classes (species) for all isolates tested. All chemometric models were performed in Matlab version 6.5

release 13 (MathWorks, Natick, MA) and the PLS Toolbox version 3.5 for Matlab (Eigenvector Research, Manson, WA).

3. Results and Discussion

In this work, we demonstrate for the first time that FTIR-ATR spectroscopy combined with PLSDA is a reliable, quick and low cost method to accurately discriminate *B. pumilus* and *B. safensis* isolates. Some previous studies used FTIR to discriminate *Bacillus* isolates at genus (Whittaker et al. 2003; Maity et al. 2013) and species (Mietke et al. 2010; Ammann & Brandl 2011; Lücking et al. 2013) levels; however none of them included the closely related species and highly relevant *B. pumilus* and *B. safensis*. Infrared spectra of the isolates included in this study revealed the presence of bands associated with bacterial components such as lipids (3000-2800 cm^{-1}), proteins/amides I and II (1700-1500 cm^{-1}), phospholipids/DNA/RNA (1500-1185 cm^{-1}), polysaccharides (1185-900 cm^{-1}) and the fingerprint region (900-600 cm^{-1}) (Naumann et al. 1991). A high degree of spectral similarity was observed among both species (Figure 1) remarking their phylogenetics (Liu *et al.,* 2013; Satomi *et al.,* 2006). Similarly to previous works (Coutinho et al., 2009; Sousa et al., 2012; Vaz et al., 2013; Sousa et al., 2014), the main spectral differences (marked with black arrows in Figure 1) were found in the phospholipids/DNA/RNA and the polysaccharides regions (1500-900cm^{-1}), which were chosen for further comparisons. Those differences reflects the cellular composition dissimilarity between both species which mainly corresponds to vibrations associated with C–H bending of alkanes (1461 cm^{-1}); $(CH_3)_3N^+$ symmetric bending (lipids) and C–H bending of alkenes $=CH_2$ (1414 cm^{-1}); C–O stretching of RNA ribose (1165 cm^{-1}); alcoholic C–OH vibration of polysaccharide fraction and $>PO_2^-$ stretching (1070 cm^{-1}); S=O stretching of organic sulfoxides (1052 cm^{-1}), P–O–P stretching (phospholipids, ribose phosphate chain pyrophosphate and glycipeptides) and C–O stretching of nucleic acids (1030-980 cm^{-1}). In addition, a PLSDA of the infrared spectra in the selected region revealed two perfectly distinct clusters, each one containing isolates of one single species (Figure 2). Moreover, model statistics (data not shown) revealed that 98.05% of the isolates perfectly fit the developed model, being the first three latent variables (that encompass 52.97% of spectral variability) responsible for species discrimination. Although the results obtained herein only include two species of the *B. pumillus* group they strongly indicated that FTIR-ATR possess a high potential in the discrimination of *Bacillus* spp. Moreover, FTIR-ATR seems to possess the same discriminatory power as other high throughput techniques such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (unpublished results) with the advantage of having a smaller cost, concerning equipment and analysis

and be totally free of reagents. The reliability of this technique for species discrimination should be further tested in a higher number of species of the *B. pumillus* group as *B. altitudinis, B. stratosphericus*, *B. aerophilus* and the newly described *B. xiamenensis* (Lai et al. 2014) as well as in a higher number of isolates of *B. pumillus* and *B. safensis*. At this moment one doesn't possess conveniently characterized isolates of *B. altitudinis* and *B. xiamenensis* and it was not possible to obtain *B. stratosphericus* and *B. aerophilus* in the type culture collections neither with the authors responsible for their first description.

4. Conclusions

We demonstrated that species-specific cellular components of *B. pumilus* and *B. safensis* isolates detected by FTIR-ATR can differentiate these closed species. This achievement is of high relevance due to the possibility to reliably differentiate isolates belonging to these closely related species rapidly and with low cost, which is of interest for quality control purposes in different industrial production units. Furthermore, this study unveils molecular signatures (e.g. carbohydrates and phospholipids) of these species that could be further explored to improve the overall knowledge of these bacteria.

Acknowledgements

This study was supported by Fundação para a Ciência e Tecnologia grants (PEst-C/EQB/LA0006/2011, POCI/AMB/61814/2004-FSE/FEDER). Clara Sousa was supported by a post-doctoral grant (SFRH/BPD/70548/2010) and Raquel Branquinho by a PhD grant (SFRH/BD/61410/2009) from Fundação para a Ciência e Tecnologia.

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Table 1. Features of *Bacillus pumilus* and *B. safensis* included in this study.

	Isolate	PFGE[a]	Origin	Year/Location	**References**
	ATCC 7061[T]	NA	----	----	Type Strain
Bacillus pumilus	Bp7	I_p	Health products (n=3)	2005/Portugal	Branquinho et al., 2012
	Bp11			2005/Portugal	
	Bp15			2005/Portugal	
Bacillus safensis	Bs1	V_s	Animals Gastropods (n=3)	2004/Portugal	Branquinho et al., 2012
	Bs2			2005/Portugal	
	Bs3			2007/Portugal	
	Bs13	VII_s	Health products (n=3)	2005/Portugal	
	Bs16	III_s		2005/Portugal	
	Bs17	IV_s		2005/Portugal	
	Bs5	XI_s	Cosmetic´s (n=4)	2002/Portugal	
	Bs18	VI_s		2002/Portugal	
	Bs27	II_s		2002/Portugal	
	Bs24	XV_s	Food´s /salame (n=3)	2004/Italy	Matarante et al., 2004
	Bs25	$VIII_s$		2004/Italy	
	Bs33	Non typeable		2004/Italy	
	Bs22	IX_s	Plant Growth Promoters (PGPR) (n=2)	1997/USA	Jetiyanon et al., 1997
	Bs23	I_s		1997/USA	
	Bs31	X_s	Food/beans (n=1)	2003/Africa	Ouoba et al., 2004
	FO-36b[T]	$XIII_s$	Clean-room/ air particulate (n=1)	1999/USA	Satomi et al., 2006 (Type Strain)
	Bs35	XIV_s	Clean-room/floor (n=1)	2001/USA	Satomi et al., 2006
	Bs37	$XIII_s$	Clean-room/Mars Odyssey spacecraft surface (n=2)	2001/USA	
	Bs38				
	Bs42	Non typeable	Clean-room/anteroom (n=1)	2001/USA	

[a]PFGE- Pulsed Field Gel Electrophoresis; NA - not applicable.

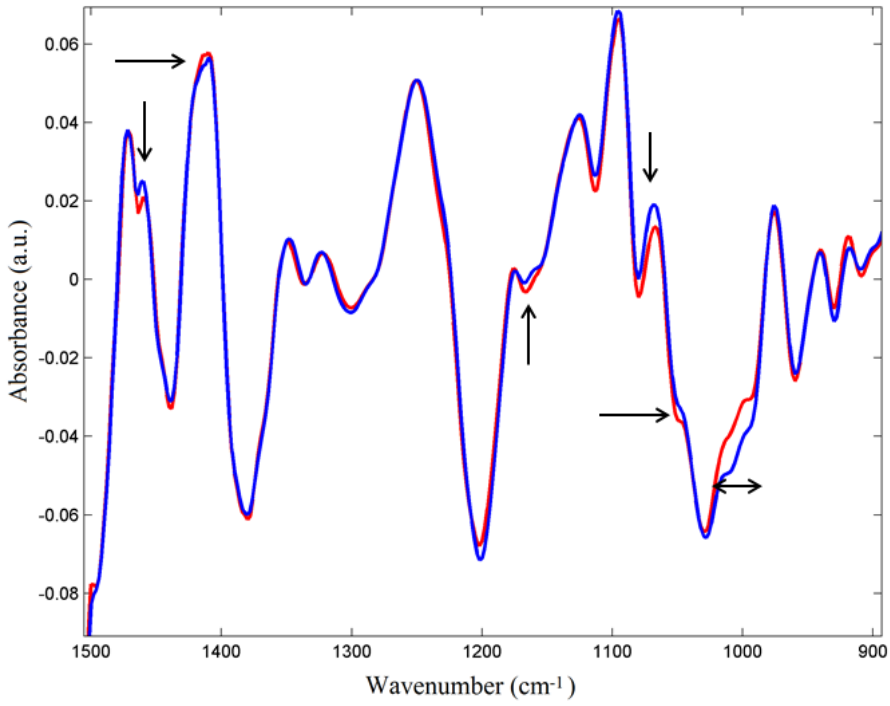

Figure 1. FTIR-ATR mean spectra of *B. pumillus* and *B. safensis* isolates processed with SNV and Savitzky-Golay (7 points filter size, 2nd degree polynomial, 1st derivative) in the region 1500-900 cm^{-1}. Legend: — *B. pumillus* and — *B. safensis*.

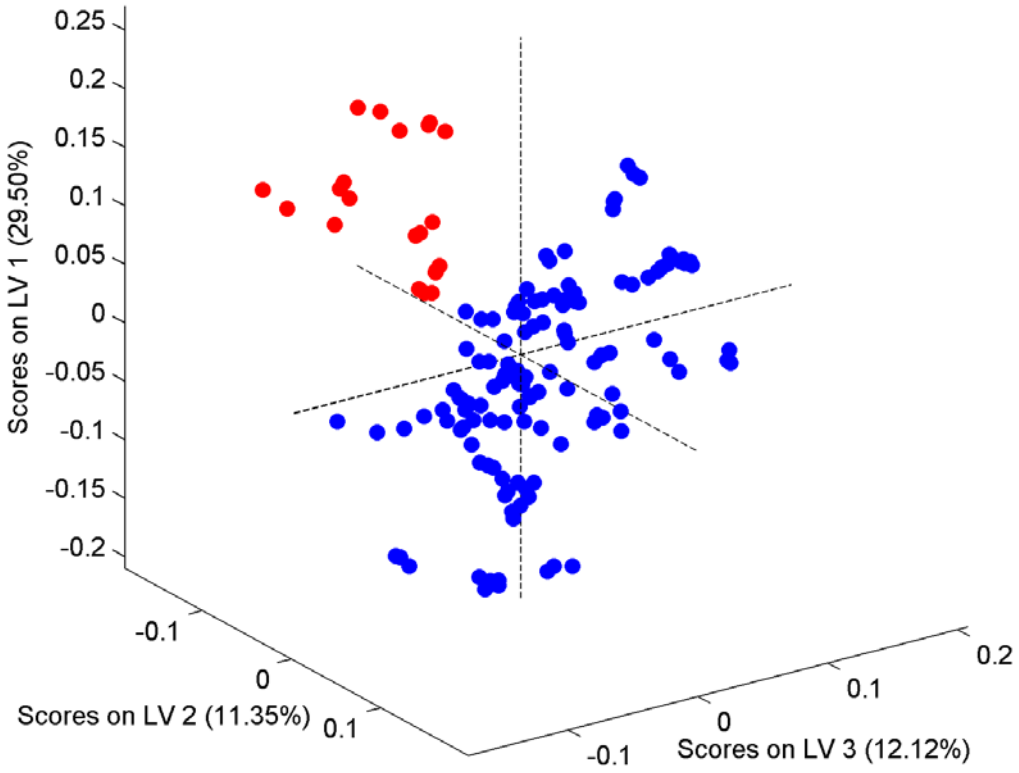

Figure 2. Score plot corresponding to the three first LVs of the PLSDA regression model using the 1500-900 cm^{-1} spectral region. Legend: ● *B. pumillus* and ● *B. safensis*.

2.5. Safety profile of *Bacillus subtilis* complex: antibiotic and virulence features

Publications:

Safety profile of *Bacillus subtilis* complex: antibiotic and virulence features

This study focus the absence of critical features for the usage of *B. subtilis* complex isolates in the food sector as recommended in FEEDAP guidelines, also enabling the extension of its application for other relevant biotechnological purposes.

Safety profile of *Bacillus subtilis* complex: antibiotic and virulence features

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Running title: Safety potential of *Bacillus subtilis* complex

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Manuscript in Preparation

The biotechnological and pharmaceutical relevance of *Bacillus subtilis* group members is mainly supported by their ability to produce compounds with different biological activities, namely antibacterial (Mannanov and Sattarova 2001; Seydlová et al 2011; Balciunas *et al*., 2013), antifungal (Gupte *et al*., 2002; Zhao *et al*., 2013), insecticide (Revathi *et al*., 2013), biosurfactant (Fracchia *et al*., 2013; Martinotti *et al*., 2013), probiotic and plant growth promoters (USEPA 2004; Pérez-García *et al*., 2011). In order to be used as feed additives, biotechnological products containing *Bacillus* species need to attain the Qualified Presumption of Safety (QPS) status (EFSA, 2013). For this purpose, EFSA-FEEDAP guidelines state that microorganisms cannot be resistant to antibiotics of human and veterinary importance, neither possess acquired antibiotic resistance genes and toxin encoding genes (EFSA, 2011).

In this study, we determined the susceptibility and presence of resistance genes to several antimicrobials, β-lactamase activity and presence of genes encoding for entero-, emetic- and cyto-toxins in a collection of *Bacillus subtilis* complex isolates, contributing for the establishment of their safety profile.

Forty-three *Bacillus subtilis* complex isolates (27 *B. safensis*, 9 *B. altitudinis*, 4 *B. pumilus* and 3 *B. subtilis*) previously identified by phenotypic and genotypic (16S rRNA, *gyrB and rpoB* gene sequences) methods, were tested (Branquinho *et al*., 2014) (Table S1). Minimum inhibitory concentrations (MIC) to different antibiotics were determined by broth microdilution method and interpreted according to clinical breakpoints (CLSI M45-A, 2006) or microbiological cut-offs (Table 1) (EFSA, 2012). All MIC experiments were performed in duplicate. β-lactamase activity was assessed through the nitrocefin test (Antunes *et al*., 2004). Transferable genes encoding resistance to tetracylines, macrolides, aminoglycosides, glycopeptides, phenicols and oxazolidinones, and toxins usually present in *B. cereus* (cytotoxin K, non-hemolytic enterotoxins, haemolysin and emetic toxins) were screened by PCR using primers and conditions outlined in Table S2.

All isolates presented MICs to the different antibiotics lower than the established microbiological cut-offs for the detection of acquired antibiotic resistance genes, suggesting their absence, which was further confirmed by the absence of resistance genes. Remarkably, isolates were clinically intermediate or resistant to cefotaxime, except for *B. subtilis* isolates. Moreover one *B. subtilis* presented MIC levels to penicillin that would prevent the therapeutical success of this antibiotic. In addition, nitrocefin test revealed that 69.7% of the isolates produced β-lactamases (18 *B. safensis*, 8 *B. altitudinis* and 4 *B. pumilus*). Furthermore, no amplification for toxins encoding toxins was obtained.

According to the FEEDAP guidelines, this collection fulfills the antibiotic resistance parameters for the QPS, as none of the isolates would apparently constitute a reservoir for mobile antibiotic resistance genes, and thus could be of potential used for industrial purposes, including as feed additives. Nevertheless, the identification of isolates with intermediate or clinical resistance to cefotaxime or penicillin (both antibiotics recommended for testing in CLSI-M45 but not required by the FEEDAP guidelines), should be considered in the strains safety evaluations due to the therapeutic failure that would be observed with their use. Despite the absence of toxins usually encoded by *B. cereus*, the assessment in these isolates of surfactin-like compounds production should be further studied.

It is widely known that *Bacillus* spp. can harbor intrinsic β-lactamases (Andrews and Wise, 2002), however their clinical importance has been poorly studied. Our results show that β-lactamase production is not correlated with higher MIC values to β-lactams, as the same MIC for ampicillin is verified either for β-lactamase producers and non-producers. Despite these observations, we should also consider these β-lactamases: i) probably possess additional/unknown factors required for their activity; and ii) might have high substrate specificity. Besides, Penicilin-Binding-Proteins (PBP) variants with different β-lactam affinities might lead to variable susceptibility behavior to these antibiotics, as previously observed for other Gram positive bacteria (Leclercq *et al*., 2013).

In conclusion, this work demonstrates the absence of antibiotic and virulence features required for the usage of *B. subtilis* complex isolates in the food sector, also enabling their safe application for other relevant biotechnological purposes. In addition, future QPS assessments should take into consideration the assessment of susceptibility to clinically relevant antibiotics, such as cefotaxime and penicillin in *Bacillus* spp.

Table 1. Susceptibility profile of *Bacillus* spp. tested.

Antibiotic	Clinical breakpoint[a] (μg/mL) (S/I/R)	Microbiological[b] cut-off (μg/mL)	Species (n)	MIC (μg/mL) range	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Nº of I and R Isolates
Penicillin	<0.12 / - / ≥0.25	NA	*B. safensis* (n=27)	<0.03	<0.03	<0.03	0
			B. altitudinis (n=9)	<0.03	<0.03	<0.03	0
			B. pumilus (n=4)	<0.03	<0.03	<0.03	0
			B. subtilis (n=3)	<0.03->64	<0.03	>64	1
Oxacillin	NA		*B. safensis* (n=27)	<0.06-0.125	0.125	0.125	NA
			B. altitudinis (n=9)	<0.06-0.125	<0.06	0.125	
			B. pumilus (n=4)	<0.06-0.125	0.125	0.125	
			B. subtilis (n=3)	<0.06	<0.06	<0.06	
Ampicillin	≤0.25 / - / ≥0.5	NA	*B. safensis* (n=27)	<0.07	<0.07	<0.07	0
			B. altitudinis (n=9)	<0.07	<0.07	<0.07	0
			B. pumilus (n=4)	<0.07	<0.07	<0.07	0
			B. subtilis (n=3)	<0.07	<0.07	<0.07	0
Amoxicillin	NA		*B. safensis* (n=27)	<0.06	<0.06	<0.06	NA
			B. altitudinis (n=9)	<0.06	<0.06	<0.06	
			B. pumilus (n=4)	<0.06	<0.06	<0.06	
			B. subtilis (n=3)	<0.06	<0.06	<0.06	
Piperacillin	NA		*B. safensis* (n=27)	0.5-2	1	1	NA
			B. altitudinis (n=9)	<0.5-1	0.5	1	
			B. pumilus (n=4)	1	1	1	
			B. subtilis (n=3)	0.25-0.5	0.25	0.5	
Cefotaxime	≤8 / 16-32 / ≥64	NA	*B. safensis* (n=27)	16-64	32	32	27
			B. altitudinis (n=9)	8-64	32	64	8
			B. pumilus (n=4)	16-32	32	32	4
			B. subtilis (n=3)	<0.125	<0.125	<0.125	0
Cefoxitin	NA		*B. safensis* (n=27)	<0.125-4	2	2	NA
			B. altitudinis (n=9)	1-4	1	1	
			B. pumilus (n=4)	1-2	2	2	
			B. subtilis (n=3)	<0.125-1	<0.125	1	
Ertapenem	NA		*B. safensis* (n=27)	0.06-0.125	0.125	0.125	NA
			B. altitudinis (n=9)	0.03-0.125	0.03	0.06	
			B. pumilus (n=4)	0.125-0.25	0.125	0.25	
			B. subtilis (n=3)	<0.016-0.03	0.03	0.03	
Imipenem	≤4 / 6 / ≥16	NA	*B. safensis* (n=27)	<0.07	<0.07	<0.07	0
			B. altitudinis (n=9)	<0.07	<0.07	<0.07	0
			B. pumilus (n=4)	<0.07	<0.07	<0.07	0

			B. subtilis (n=3)	<0.07	<0.07	<0.07	0
Vancomycin	≤4 / - / -	4	*B. safensis* (n=27)	<0.07-0.25	0.125	0.125	0
			B. altitudinis (n=9)	<0.07-0.25	0.125	0.25	0
			B. pumilus (n=4)	0.125	0.125	0.125	0
			B. subtilis (n=3)	0.125	0.125	0.125	0
Gentamicin	≤4 / 8 / ≥16	4	*B. safensis* (n=27)	<0.07	<0.07	<0.07	0
			B. altitudinis (n=9)	<0.07	<0.07	<0.07	0
			B. pumilus (n=4)	<0.07	<0.07	<0.07	0
			B. subtilis (n=3)	<0.07	<0.07	<0.07	0
Amikacin	≤16 / 32 / ≥64	NA	*B. safensis* (n=27)	<0.06-0.5	0.125	0.125	0
			B. altitudinis (n=9)	<0.06-0.25	<0.06	0.125	0
			B. pumilus (n=4)	<0.06-0.125	0.125	0.125	0
			B. subtilis (n=3)	0.25-0.5	0.25	0.5	0
Kanamycin	NA	8	*B. safensis* (n=27)	0.125-0.5	0.25	0.25	0
			B. altitudinis (n=9)	0.125-0.5	0.25	0.25	0
			B. pumilus (n=4)	0.125-0.25	0.25	0.25	0
			B. subtilis (n=3)	0.25-0.5	0.25	0.5	0
Streptomycin	NA	8	*B. safensis* (n=27)	1-2	1	2	0
			B. altitudinis (n=9)	1-2	2	2	0
			B. pumilus (n=4)	2	2	2	0
			B. subtilis (n=3)	1-4	1	4	0
Erythromycin	≤0.5 / 1-4 / ≥8	4	*B. safensis* (n=27)	<0.016-0.06	0.03	0.06	0
			B. altitudinis (n=9)	<0.016-0.03	<0.016	<0.016	0
			B. pumilus (n=4)	0.03-0.06	0.03	0.06	0
			B. subtilis (n=3)	<0.016-0.03	0.03	0.03	0
Tetracycline	≤4 / 8 / ≥16	8	*B. safensis* (n=27)	<0.06	<0.06	<0.06	0
			B. altitudinis (n=9)	<0.06	<0.06	<0.06	0
			B. pumilus (n=4)	<0.06	<0.06	<0.06	0
			B. subtilis (n=3)	<0.06	<0.06	<0.06	0
Chlortetracycline	NA		*B. safensis* (n=27)	<0.125	<0.125	<0.125	
			B. altitudinis (n=9)	<0.125	<0.125	<0.125	NA
			B. pumilus (n=4)	<0.125	<0.125	<0.125	
			B. subtilis (n=3)	<0.125	<0.125	<0.125	
Ciprofloxacin	≤1 / 2 / ≥4	NA	*B. safensis* (n=27)	<0.03-0.06	<0.03	<0.03	0
			B. altitudinis (n=9)	<0.03	<0.03	<0.03	0
			B. pumilus (n=4)	<0.03	<0.03	<0.03	0
			B. subtilis (n=3)	<0.03	<0.03	<0.03	0
Norfloxacin	NA		*B. safensis* (n=27)	0.125-1	0.25	0.5	
			B. altitudinis (n=9)	0.125-0.5	0.25	0.5	NA
			B. pumilus (n=4)	0.25-0.5	0.25	0.5	

			B. subtilis (n=3)	<0.07-0.125	<0.07	0.125	
Florphenicol	NA		*B. safensis* (n=27) *B. altitudinis* (n=9) *B. pumilus* (n=4) *B. subtilis* (n=3)	1-2 1-2 2 1	2 1 2 1	2 2 2 1	NA
Tiamulin	NA		*B. safensis* (n=27) *B. altitudinis* (n=9) *B. pumilus* (n=4) *B. subtilis* (n=3)	>128 1->128 >128 >128	>128 >128 >128 >128	>128 >128 >128 >128	NA
Rifampin	≤1 / 2 / ≥4	NA	*B. safensis* (n=27) *B. altitudinis* (n=9) *B. pumilus* (n=4) *B. subtilis* (n=3)	<0.016 <0.016 <0.016 <0.016	<0.016 <0.016 <0.016 <0.016	<0.016 <0.016 <0.016 <0.016	0 0 0 0

[a] CLSI - Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria – Standard M45-A 2006; [b]FEEDAP - Scientific opinion on guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance; NA – not applicable

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Supporting Information

Safety profile of *Bacillus subtilis* complex: antibiotic and virulence features

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Running title: Safety potential of *Bacillus subtilis* complex

Table S1. *Bacillus subtilis* complex isolates included in this study.

Species	Isolates	Origin	Year/Location	References
Bacillus pumilus (n=4)	ATCC 14884	----	----	Reference Strain
	Bp7 Bp11 Bp15	Health´s products (n=3)	2005/Portugal[1]	Branquinho et al., 2012
Bacillus altitudinis (n=9)	Ba6 Ba8 Ba9 Ba12 Ba14	Health´s products (n=5)	2005/Portugal[1]	Branquinho et al., 2012
	Ba26	Food´s contaminants (n=1)	2006/Norway	From et al 2007
	Ba30	Food (n=1)	2003/Africa	Ouoba et al 2003
	Ba20	Plant Growth Promoters (PGPR) (n=2)	1996/USA	Wei et al 1996
	Ba21		1997/USA	Jetiyanon et al 1997
Bacillus safensis (n=27)	Bs1	Animals Gastropods (n=3)	2004/Portugal[1]	Branquinho *et al*., 2012
	Bs2		2005/Portugal[1]	
	Bs3		2007/Portugal[1]	
	Bs13 Bs16 Bs17	Health´s products (n=3)	2005/Portugal[1]	
	Bs5 Bs18 Bs19	Cosmetic´s (n=4)	2002/Portugal[1]	
	Bs27 Bs24 Bs25 Bs33	Food´s contaminants (n=3)	2004/Italy[2]	Matarante *et al*., 2004
	Bs22 Bs23	Plant Growth Promoters (PGPR) (n=2)	1997/USA[3]	Jetiyanon *et al*., 1997
	Bs31	Food (n=1)	2003/Africa[4]	Ouoba *et al*., 2004
	FO-36b^T	Clean-room/ air particulate (n=1)	1999/USA[5]	Type Strain, Satomi *et al*., 2006
	Bs32 Bs34 Bs35	Clean-room/floor (n=3)	2001/USA[5]	Satomi *et al*., 2006
	Bs36	Clean-room/cabinet top (n=1)		
	Bs37 Bs38 Bs39 Bs40	Clean-room/Mars Odyssey spacecraft surface (n=5)	2002/USA	

	Bs41 Bs42	Clean-room/anteroom (n=1)	2001/USA[5]	
	Bsb28	Health´s products (n=1)	2005/Portugal[1]	This study
Bacillus subtilis (n=3)	Bsb_{FFUP1}	----	----	FFUP culture collection, this study
	Bsb_{BGA}	----	----	This study

Table S2. Primers and amplification conditions used for detection of enterotoxin and resistance genes

	Target gene	Primer name	Sequence	Amplicon size (bp)	Annealing Temperature (º C)	Reference
	Emetic and cytotoxin genes					
Emetic toxin	*cesA*	CER1_Fw EMT1_Rv	ATCATAAAGGACAAGA AAGATCAACCCAACTG	188	52	Ehling-Schulz *et al*., 2004
	cesB	EM1_Fw EM1_Rv	GACAAGAGAATACAAT GCAGCCTTCCCACAGT	635	60	Ehling-Schulz *et al*., 2004
Cytotoxin	*cytK*	FC_Fw RC_Rv	GTAACTTTCATTTGATGATC GAATACATAAATAATTGGT	505	50	Ehling-Schulz *et al*., 2004
	hblC	L2_Fw L2_Rv	AGAAACTCAACAAGAAAACATGG TTGCGCAGTTGCCACATTAG	444	50	Ehling-Schulz *et al*., 2004
	nheA	0045_Fw 4091_Rv	GTTAGGATCACAATCACCGC CCATATGCATTTGTAAAATCTG	264	50	Ehling-Schulz *et al*., 2004
	nheB	517_Fw 8368_Rv	CGGTTCATCTGTTGCGACAGC GATCCCATTGTGTACCATTGG	335	50	Ehling-Schulz *et al*., 2004
	nheC	4924_Fw 1141_Rv	GCGATTGATCAAAAGGATAG CGACTTCTGCTTGTGCTCCTG	411	50	Ehling-Schulz *et al*., 2004
	Resistance genes					
Tetracycline	*tet M*	tetM-F tetM-R	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	657	53	Aarestrup *et al*., 2000.
	tet L	tetL-F tetL-R	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG	475	53	Aarestrup *et al*., 2000.

	tet S	tetS-F tetS-R	TGGAACGCCAGAGAGGTATT ACATAGACAAGCCGTTGACC	661	56	Aarestrup *et al*., 2000.
	tet K	tetK-F tetK-R	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	718	56	Aarestrup *et al*., 2000.
	tet O	tetO-F tetO-R	CAATATCACCAGAGCAGGCT GATGGCATACAGGCACAGAC	634	53	Aarestrup *et al*., 2000.
Macrolides	*ermA*	ermA1-F ermA2-R	CTTCGATAGTTTATTAATATTAGT TCTAAAAAGCATGTAAAAGAA	645	50	Sutcliffe *et al*., 1996
	ermB	ermB1-F ermB2-R	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	402	50	Sutcliffe *et al*., 1996
	ermC	ermC1-F ermC2-R	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	642	50	Sutcliffe *et al*., 1996
Glycopeptides	*vanA*	vanA-F vanA-R	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	54	Dutka-Malen *et al*., 1995
	vanB	vanB-F vanB-R	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	54	Dutka-Malen *et al*., 1995
Aminoglicosydes	*aph(2'')-Ib*	aph(2")-IbF aph(2")-IbR	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCC TT	867	52	Vakulenko *et al*., 2003
	aph(2'')-Id	aph(2")-IdF aph(2")-IdR	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	641	52	Vakulenko *et al*., 2003
	aph(2'')-Ic	aph(2")-IcF aph(2")-IcR	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG	444	52	Vakulenko *et al*., 2003

	aph(3'')-IIIa	aph(3'')-IIIaF aph(3'')-IIIaR	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	52	Vakulenko *et al.*, 2003
	aac(6')-Ie-aph(2'')-Ia	aac(6')-Ie-aph(2'')-IaF- aac(6')-Ie-aph(2'')-IaR	CATTATACAGAGCCTTGGGAAGATG GTCTTAAAAAACTGGCAATATCTCGTT	778	52	Vakulenko *et al.*, 2003
Phenicols	*fexA*	fexA-F fexA-R	GTACTTGTAGGTGCAATTACGGCTGA CGCATCTGAGTAGGACATAGCGTC	1272	58	Kehrenberg and Schwarz 2006
	floR	floR_F floR_R	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	868	55	Guerra et al. 2004
Phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A	*cfr*	cfr-fw cfr-rv	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	746	48	Kehrenberg and Schwarz 2006

CHAPTER 3

Characterization of the antibacterial compound produced by *Bacillus safensis* Bs1, the medicine-slug isolate

3.1. Characterization of the antibacterial compound produced by *Bacillus safensis* Bs1

Publication:

Characterization of a new antimicrobial peptide with anti-MRSA activity produced by *Bacillus safensis* Bs1

Results disclosed from this work proved the hypothesis proposed for this thesis, in which clonal lineage adapted to an *Arion ater, Bacillus safensis* Bs1, revealed the production of a potentially novel cationic peptide (48-residues) with anti-staphylococci activity.

Characterization of a new antimicrobial peptide with anti-MRSA activity produced by *Bacillus safensis* Bs1

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Keywords: *Bacillus safensis*, Antimicrobial peptide, SDS-PAGE, MALDI-TOF MS-MS, N-terminal sequencing, CD, AMPA

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Abstract

We previously demonstrated the, *in vitro*, anti-staphylococci activity of a *Bacillus safensis* strain (Bs1) recovered from a slug used in the treatment of cutaneous infections. In the present work the characterization of an antimicrobial peptide produced by *B. safensis* Bs1 is presented.

The Bs1 antimicrobial peptide was recovered and purified by a combination of SDS-PAGE, ultrafiltration, ammonium sulfate precipitation and chromatographic approaches. The integrity of the purified peptide was verified by mass spectrometry and N-terminal sequencing and prediction of its antimicrobial stretches was also attempted, using AMPA and CAMP databases.

The performed characterization revealed a new cationic peptide (48-residues) with antimicrobial activity against Gram-positive bacteria, including (Methicillin-resistant *Staphylococcus aureus*) MRSA. No remarkably amino acid sequence homologies were detected with other classes of antimicrobial peptides previously described. Further studies should be conducted in order to confirm the proposed amino acid composition and also its structure elucidation. Moreover, its antimicrobial property against a panel of clinical isolates expressing different mechanisms of resistance should also be extended.

1. Introduction

Due to the decline in antibiotic discovery, illustrated by a low number of new registered entities and a dramatic lack of new antibiotic classes, the increasing of multi-drug resistant bacteria causing infection is a serious concern (Carlet *et al*., 2012)**.** *Staphylococcus aureus* is one of the most disturbing and common human pathogens, being the most frequent agent of skin and soft tissues infections, osteomyelitis and bacteremia (Elsayed *et al*., 2004; Weigelt *et al*., 2005). Higher morbidity and mortality in this species is associated with methicillin-resistant *S. aureus* (MRSA) which often are also resistant to diverse antibiotic classes (Appelbaum *et al*., 2007).

In fact, in the last decades several pre-existing molecules have been modified in new derivatives, in an attempt to circumvent bacterial resistance mechanisms. Nevertheless, bacteria have demonstrated the potential to create new mechanisms of resistance allowing its surviving due to mutations or acquisition of resistance genes (Martínez and Baquero, 2002). It was only recently that linezolid, belonging to oxazolidinones class, was introduced in therapeutic use. Afterward, daptomycin and tygecicline also with activity upon MRSA, were introduced in therapeutic armamentarium. Nevertheless, and despite their recent introduction, linezolid and tygecycline resistant isolates have already emerged during treatment of *S. aureus* infections (Hentschke *et al*., 2008) being therefore imperative the recognition of therapeutic alternatives.

Therapeutic applications of organic compounds from terrestrial microorganisms have an extensive past and present use in the treatment of diseases and serve as agents of interest both in their natural form and as templates for synthetic modification. In fact, secondary metabolites from natural sources and related drugs are used to treat 87% of all categorized human diseases, including antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressive agents (Chin *et al*., 2006; Raja *et al*., 2010).

The great number of antibiotics occurs as a result of secondary metabolic pathways and can be produced by different microorganisms with a diverse chemical nature, and such diversity is reflected in its mode of action (Stein, 2005).

Among the most thoroughly studied secondary metabolites with remarkable therapeutic effects are antimicrobial peptides (AMPs) agents. Although their sequences vary, in general, they present an overall positive charge, an amphipathic structure and comprised of 10–50 amino acids, among which cysteine, lysine, proline or arginine are key compositions (Agerberth *et al*., 1991; Sahl *et al*., 2005; Groot *et al*., 2006; Lesmes *et al*.,

2009; Scocchi *et al*., 2011). Moreover, under hydrophobic environment, AMPs can fold into four classes of structures, including α-helix, β-sheets, extended structures, and loops (Brown and Hancock 2006; Peters *et al*., 2010). Related to its mechanisms of action, it is well known that most of them interact with bacterial membranes (Melo *et al*., 2009; Peters *et al*., 2010).

In the case of *Bacillus* spp., AMPs compounds are subdivided into different classes, comprising bacteriocins (Oscáriz and Pisabarro 2000; Bizani and Brandelli 2002; Cherif *et al*., 2001, 2003 and 2008; Abriouel *et al*., 2011), bacteriocin-like inhibitory substances (BLIs) (Korenblum *et al*., 2005; Cladera-Oliveira *et al*., 2004; Aunpad and Na-Bangchang 2007; Xie *et al*., 2009), polyketides (PKs) (Chen *et al*., 2007; Arguelles-Arias *et al*., 2009), nonribosomal peptides (NRPs) (Naruse *et al*., 1990; Peypoux *et al*., 1999; Hathout *et al*., 2000; Bonmatin *et al*., 2002; Lee *et al*., 2007; Caboche *et al*., 2008; Rodrigues and Teixeira 2010; Nerurkar *et al*., 2010), miscellaneous antibiotics (Tamehiro *et al*., 2002; Pinchuk *et al*., 2002; Inaoka and Ochi 2007) and unusual peptides (Kino *et al*., 2009 and 2010; Borisova *et al*., 2010)

Therefore, and considering the enormous diversity of *Bacillus* spp. in nature and the abundance of chemical structure compounds that they are able to produce, reflected also on its biological activities (Abriouel *et al*., 2011), they clearly represent an interesting challenge to explore.

During several decades, in some regions of Portugal, a post mortem terrestrial black slug was used as traditional medicine in the treatment of cutaneous infections, including wounds and abscesses. Recently, we proved *in vitro*, a high anti-staphylococci activity, including in MRSA, of this slug (Branquinho, 2007). Preliminary data suggests the contribution of a peptide compound produced by a *Bacillus safensis* isolate (Branquinho et al 2014) for the observed antimicrobial activity. The high anti-MRSA activity and the therapeutic success reported for this traditional medicine together with the absence of description of secondary effects justifies the characterization of the bioactive compound(s) associated with this traditional medicine. Although some peptides with antibacterial activity have been described among *B. pumillus* group members, none was yet attributed to *B. safensis*.

Thus, in the present work, throughout the use of advanced analytical and molecular methodologies we characterized the anti-bacterial compound produced by *B. safensis* Bs1 in a perspective of applicability to the pharmaceutical, biomedical and/or food sectors.

2. Material and Methods

2.1. Bacterial strain

B. safensis isolate Bs1 recovered from a black slug normal flora, which was collected from Northern of Portugal, was evaluated in its potential to produce antimicrobial compounds. Species identification was assessed by phenotypic, biochemical and genotypic (16S rRNA, *gyrB and rpoB* gene sequences) analysis (Branquinho et al 2014). Bacterium was maintained as stock culture at − 80 °C in Tryptic Soy Broth (TSB) (BD, Germany) supplemented with 20% (v/v) glycerol.

2.2. Production of AMP´s

Pre-inoculum of *B. safensis* Bs1 isolate was prepared from Tryptic Soy Agar (TSA, BD, Germany) plates incubated overnight at 37 ºC. Their subculture was prepared under agitation at 250 rpm in 100 mL of TSB (TSB, BD, Germany) at pH=7.0. Then, for the inoculum preparation, 10 mL of pre-inoculum culture was transferred to 500 mL of TSB previously diluted (1:2) with sterile water and incubated for 24 h at 37 ºC, under agitation conditions (250 rpm). Finally, cells were separated by centrifugation at 8000 rpm during 20 min at 4 °C. Supernatants were then filtered through 0.45 μm pore-size filter (Millipore Corp., Bedford MA) to obtain a cell-free supernatant (CFS) for evaluation of the activities in the further tasks.

2.3. Screening for antimicrobial activity

CFS was screened for its antimicrobial activity, by the agar well-diffusion method, against indicator strains summarized in Table 1. Briefly, indicator strains suspensions adjusted at 0.5 McFarland were swabbed in Mueller Hinton (MH) agar cation adjusted (bioMérieux, France) plates, where 5 mm diameter wells were cut and filled with 50 μL of CFS. Plates were then incubated at the optimal temperature and incubation times and inhibition zone diameters measured. Un-inoculated TSB broth was used as negative control. The experiment was conducted in triplicate on separate conditions.

2.4. Purification of AMPs

2.4.1. Ultrafiltration and precipitation of proteins with ammonium sulfate (partial purification)

Bs1 CFS were firstly concentrated by sequencial ultrafiltration using a 10-kDa and 3-kDa Millipore membrane cut-off (Millipore Corp., Bedford MA) and stored at –20 °C until use. Proteins were precipitated from 100 ml of the ultrafiltrated Bs1CFS, stirring constantly for 30 min at 4 °C to reach 20, 40, 60 and 80% (w/v) $(NH_4)_2\ SO_4$ (ammonium sulfate) (Sigma-Aldrich, St. Louis, USA) saturation. Precipitated proteins were collected by centrifugation at 10000 *g* for 30 min at 4 ºC, dissolved in a 1/20 (v/v) phosphate buffer (0.02 mol l^{-1}, pH 6.8), and dialyzed to remove ammonium sulfate. The dialysates were then lyophilized and dissolved in phosphate buffer. Finally, the activity of the purified protein(s) was tested using the well diffusion test against *S. aureus* ATCC 29213 as the indicator strains, as previously described.

2.4.1.1. Direct in-gel detection of antimicrobial activity and estimation of its molecular weight

Tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Tricine SDS-PAGE) was performed for antimicrobial peptide band identification using 16% polyacrylamide gel following the procedure of Schägger and Jagow (Schägger 2006). Twenty microliters of ultrafiltered fractions, with confirmed antimicrobial activity against *S. aureus* ATCC 29213 and direct Bs1 CFS were applied and separated onto the polyacrylamide gel, in duplicate. After electrophoresis, a part of the gel was stained with Coomassie Brilliant Blue (0.1% Coomassie R-250, 40% ethanol, 10% acetic acid) for 1 h, de-stained for 1 h (7.5% acetic acid, 10% ethanol) and washed during 4 h in sterile distilled water to visualize the protein and/or peptide bands. Moreover, the unstained part of the gel was used for *in situ* detection of AMP activity upon fixing it in a mixture of 2-propanol, acetic acid and H_2O (25:10:65) for 15 min, followed by washing with sterile water for 30 min, repeatedly four times. The SDS-PAGE gel lines were then placed in sterile Petri dishes and overlaid with 10 ml of nutrient agar (0.7%) containing *S. aureus* ATCC 29213 as the indicator strain (0.5 McFarland adjusted). Plates were finally incubated during 24 h at 37 ºC and diameter zones around the bands visualized.

2.4.2. FPLC and HPLC purification

Fractions obtained by ultrafiltration, ammonium sulfate precipitation and direct Bs1 CFS were purified by fast protein liquid chromatography (FPLC) and reverse-phase high-performance liquid chromatography (RP-HPLC).

Initial purification was accomplished using a size-exclusion FPLC system (Pharmacia Biotech, Uppsala, Sweden) with a Superose 12/HR10 column, Amersham Pharmacia Biotech) eluted with phosphate buffer (50 mM Na_2HPO_4 and 50 mM NaH_2PO_4) pH 7.0 at a flow rate of 0.5 ml min^{-1} and monitored by UV absorption at 280 nm.

Further purification by RP-HPLC system was achieved using a C18 column (5 µm, 250×4.6 mm, Phenomenex, USA). Chromatographic separations were conducted under a linear gradient of two mobile phases, A, 0.1% Trifluoroacetic Acid (TFA) in H_2O, and B, 0.1% TFA in 100% acetonitrile. Proteins were eluted over 90 min with 20-80% B gradient at a flow rate of 1 ml min^{-1} and monitored at 220 nm and 280 nm in a diode array detector.

All FPLC and HPLC eluted fractions were collected, concentrated by lyophilization and antimicrobial activity was screened by agar well-diffusion as previously detailed.

2.5. Characterization of the purified AMP

2.5.1. Mass spectrometry and peptide mass fingerprint (PMF) analysis

Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) was used to primarily characterize the purified AMP which was excised from bands demonstrating antibacterial activity against *S. aureus* ATCC 29213 recovered directly by SDS-PAGE**.**

Selected bands were subjected to digestion with trypsin (Promega). Briefly, the protein gel bands were washed with water, destained with methanol/50 mM NH_4HCO_3 and acetonitrile/50 mM NH_4HCO_3 (1:1 v/v), reduced with 25 mM DTT in 50 mM NH_4HCO_3 for 20 min at 56 °C, alkylated with 55 mM IAA in 50 mM NH_4HCO_3 for 20 min in the dark, and in-gel digested with 10 µL of 2 ng/µL trypsin for 3 h at 37 °C in the presence of 0.01% surfactant (ProteaseMAX, Promega). Finally, the resulting peptides were extracted with 20 µL of TFA 2.5% for 15 min. Protein digests were desalted, concentrated, and spotted onto

a MALDI plate using C18 ZipTips (Millipore Corp., Bedford MA) following the manufacturer's instructions.

For the matrix preparation, a solution of 6−8 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA was used. Samples were analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (AB SCIEX, Framingham, MA). Peptide mass fingerprint (PMF) data were collected in positive MS reflector mode in the range of *m/z* 1000−10000 and was calibrated with external standards and internally calibrated using trypsin autolysis peaks. The MS and MS/MS spectra were processed and analyzed using the software GPS Explorer (Version 3.6, AB SCIEX, Framingham, MA) and were searched together against the UniProt protein sequence database using the Mascot search engine (Version 2.1.04, Matrix Science, UK) limited to Firmicutes taxonomy. During database search the following parameters were considered: database, maximum missed cleavages, 1; precursor tolerance, 0.2 Da; peptide charges, +1; mass, monoisotopic.

2.5.2. N-terminal sequencing

The purified BS1 peptide was also analyzed by automated N-terminal sequencing in order to validate the MS identification. The peptide was previously transferred onto a polyvinylidene fluoride membrane (PVDF) (BioRad CA, USA) and then excised and subjected to automated Edman degradation using a 492 Procise protein sequencer (Applied Biosystems, Foster City, CA).

2.5.3. Secondary structure prediction

Circular dichroism (CD) measurements of the purified peptide were performed on a Jasco J-815 (Jasco Inc.) equipped with a Peltier temperature-controlled cell holder, at 20 º C, in the far UV region (190-260 nm) using a path length cuvette (0.2-mm optical path-length). Prediction of the protein secondary structure content was estimated using the DichroWeb server and Greenfield and Fasman method (Greenfield and Fasman, 1969).

2.5.4. Detection of possible post-translational modifications: glycosylation

The presence of glycosylated proteins was evaluated directly in SDS-PAGE gel, stained by the periodic acid-Schiff (PAS) method using the Pierce (Rockford, IL) glycoprotein staining kit, which specifically detects glycosylated proteins with sialic acid and other oxidizable carbohydrate groups by the presence of magenta bands with a light pink or colorless background.

2.5.5. Prediction of protein antimicrobial regions

For prediction of sequenced peptide antimicrobial region, and automated web server Antimicrobial Sequence Scanning System (AMPA) (http://tcoffee.crg.cat/apps/ampa), was used (Torrent *et al*., 2012). This web application uses an antimicrobial propensity scale derived from high-throughput screening, that generate an antimicrobial profile and an antimicrobial index (AI), which can be calculated providing a fair assessment of the tendency for a specific amino acid to be present in an AMP sequence. As low half maximal inhibitory concentration (IC50) values correspond to high activity, amino acids with a low index are the most favored to be part of an AMP. Moreover, in order to confirm results disclosed by AMPA and compare important chemical proprieties of our peptide, Collection of Anti-Microbial Peptides (CAMP) database (http://www.camp.bicnirrh.res.in/) was also applied (Thomas *et al*., 2010).

3. Results and Discussion

During a screening for new antimicrobial compounds in natural sources, we verified that a *B. safensis* isolate, Bs1, recovered from the normal flora of a terrestrial black slug, which was commonly used as traditional medicine, produced a mixture of compounds with a proteinaceous nature, showing a remarkably high anti-staphylococci activity (including MRSA) (Branquinho, 2007). The observation of this anti-bacterial activity could justify the reported therapeutic success of this traditional medicine applied in the treatment of wounds and abscesses. Moreover, the absence of reported adverse effects prompts us to further characterize the target compound. In the present work, antibacterial compound purification was accessed by a combination of ultrafiltration, ammonium sulfate precipitation, chromatographic analysis and SDS-PAGE. Additionally, the integrity of the purified peptide(s) was verified by mass spectrometry analysis and N-terminal sequencing. Finally, peptide secondary structure prediction, detection of the post-translational modification (glycosylation) and prediction of its antibacterial stretches were also attempted.

3.1. Screening for antimicrobial activity

The influence of different carbon and nitrogen sources, optimum pH and incubation temperature on Bs1 AMP production was previously tested (Branquinho, 2007). Therefore, TSB was selected as the best medium for its production at 37 ºC and pH 7.0±0.2. In addition, the highest AMP in late stationary *B. safensis* Bs1 growth phase (Branquinho, 2007).

This study revealed that in the presence of CFS from *B. safensis* Bs1 Gram-positive bacteria such as *S. aureus*, MRSA and *Bacillus subtilis* were inhibited (presenting inhibition zones of 14 mm, 13 mm and 12 mm respectively), and none activity was revealed against the Gram-negative bacteria tested. Moreover the antibacterial compound did not inhibited *B. pumilus*, the species phylogenetically more closely related with *B. safensis*. These results suggest a selective antibacterial activity towards some Gram positive bacteria.

3.3. Antimicrobial activity of the partial purified fractions

Ultrafiltered fractions were partially purified using ammonium sulfate to obtain 20, 40, 60 and 80% of saturation. The highest inhibitory activity against *S. aureus* ATCC 29213 was observed in the resolved precipitate with 40 and 60% of saturation (Figure 1) and the remaining fractions did not exhibit any effect against this indicator strain. In addition, it was verified that after ammonium sulfate precipitation, inhibition zones increased, when compared with direct Bs1 CFS, confirming its concentration. Therefore, both active precipitates were analyzed by SDS-PAGE (Figure 2) which revealed the presence of one isolated protein band with an apparent molecular weight of approximately 5 kDa.

3.4. Direct in-gel detection of antimicrobial activity and estimation of its molecular weight

The Bs1 CFS was concentrated through sequential ultrafiltration using a 10 kDa and 3 kDa membranes, following non denaturing electrophoresis, demonstrated the presence of several proteins (Figure 3). Moreover, overlaying the gel with nutrient agar containing *S. aureus* ATCC 29213 as indicator strain, an apparent single protein band seems to be associated with the detected antimicrobial activity (Figure 3), with an apparent molecular weight of approximately 5 kDa. Interestingly, the protein band resolved by ammonium sulfate precipitation (Figure 2) showed the same estimated molecular weight of this AMP detected directly in-gel for its antimicrobial activity.

In fact, *Bacillus* spp. appears to be an abundant source of antimicrobial compounds, since many species of this genus demonstrate the capability to synthesize antimicrobial peptides (Stein 2005). In the scientific literature, some studies revealed the production of AMP agents with an apparent similar molecular weight of that observed in the present study. Therefore, Cherif *et al*., (2008) reported a 4.8 kDa bacteriocin-like substance (BLIS) produced by *Bacillus thuringiensis* subsp. *entomocidus*, Lisboa *et al*., (2006) found a 5 kDa AMP in a *Bacillus* spp., Xie *et al*., (2009) described a BLIS produced by *Bacillus subtilis* LFB112 with an apparent molecular weight of 6.3 kDa, Alam *et al*., (2011) reported a BLIS produced by *B. subtilis* strain BS15 with 3-5 kDa, Baindara *et al*., (2013) from a halotolerant *B. subtilis* strain SK.DU.4 described a AMP with 5323.9 Da and finally Compaoré *et al*., (2013) disclosed a 4kDa compound produced by a *B. subtilis* subs. *subtilis* strain H4. Nevertheless, characterization of these compounds, namely on its

amino acid compositions and correspondent structures elucidation was poorly explored, which hinders comparisons.

3.5. Antimicrobial activity of the FPLC and HPLC purified fractions

In order to separate the active Bs1 peptide, a combination of chromatographic techniques were applied and the fractions obtained tested for its antimicrobial activity against *S. aureus* ATCC 29213 using well diffusion assays. In both FPLC and HPLC analysis, the presence of active peptide in the different Bs1 CFS extracts was initially confirmed. Resulting chromatograms (Figure S1 and S2) beside the active peptide, seems to present a large number of other peptides and proteins.

FPLC elution profiles analysis of ultrafiltered fractions with 10 kDa and 3 kDa membranes, followed by ammonium sulfate precipitation at 40% and 60% of saturation (Figure S1), showed the presence of an additional peak (when compared with the negative control), in the retention time between 50 and 60 min, which possibly corresponds to the previously associated active peptide with 5 kDa. After fractions collection, a lyophilization step was preceded and then the antimicrobial activity tested. Unfortunately, no fraction exhibited antimicrobial activity against the strain indicator tested.

HPLC chromatograms analysis of the Bs1 active extracts (Figure S2) revealed the presence of distinct peaks (when compared with the negative control), after 35 min of retention time. Moreover, and under these conditions different inactive peptides seem to be also precipitated (Figure S2 (d) and (e)). All fractions derived from ammonium sulfate precipitation were collected, lyophilized and antimicrobial activity of each individual peak was assessed. Similar to FPLC, no fraction presented antimicrobial activity against the indicator organism tested.

Thus, in order to evaluate the presence of the active peptide in the correspondent pre-purified steps prior to structural characterization, fractions derived from these steps were also purified by C18 ZipTip and the single collected fraction tested for its antimicrobial activity. The resulting purified peptide from both 40% and 60% of ammonium sulfate precipitation revealed activity against *S. aureus* ATCC 29213, with inhibition zones of 18 mm detected by well diffusion assay.

These results prompt us to suggest that possibly the chromatographic separation induced a structural change in the active peptide. Thus, the absence of antibacterial activity in the

fractions recovered from FPLC and HPLC can be probably associated to the combination of some essential peaks, which cooperatively functionalize the activity of peptide and actively changed during HPLC and FPLC separation. Chromatographic separations can cause irreversible denaturation of peptide, thereby reducing the potential of their recovery in a biologically active form (Aguilar, 2003). Indeed, the nature of the organic solvent can influence the peptide conformation and also, the use of longer gradient times, despite provides improved separation, can also increase the residence time of the peptide at the sorbent surface, which may then result in an increase of the peptide denaturation degree (Aguilar 2003). Therefore, this hypothesis could possible justify the results obtained, since they can confer an impact on the level of recovery of biologically active material or impose important changes in peptide structure.

3.6. Characterization of the purified AMP

3.6.1. Mass spectrometry and PMF

Mass spectrometry analysis of the excised SDS-PAGE gel bands, resolved with 40% and 60% of ammonium sulfate saturation, which revealed the presence of an antimicrobial peptide with approximately 5 kDa, demonstrate the presence of two ion peaks at *m/z* of 5372.6 and 5502.5 (Figure 4). This difference probably corresponds to the Met losses (-131 Da) in the *m/z* 5372.6.

Further, primary structure elucidation of these *m/z* peaks, conducted using their tryptic digestion, was accesses by MS/MS. The resulting digestion revealed the presence of two main fragment peptides (Figure 5) at *m/z* 1540.75 and 1991.87, corresponding respectively to SAPTCFLQGVGTFR and YYVDRDTGEICTSQER. PMF analysis was performed by Matrix Science Mascot UK software, and significant ($P<0.05$) result was obtained. After searching in the MASCOT protein database for amino acid sequences identity, the partially resulted sequence, matches the sequence of a hypothetical protein including 64 amino acids, which was derived from the genome of *B. pumilus* ATCC 7061^T (gi 489307730), whose complete sequence is MLDRFLNKMTNVQPAASRCILQKLVTSCKSAPTCFLQGVGTFRYYVDRDTGEICTSQERI RCGC. Our partial sequence identified, present 100% of homology with 30 amino acids of the previous one, from position 30 to 59 (SAPTCFLQGVGTFRYYVDRDTGEICTSQER). Except for the amino sequence, no any other information such as function, genetic location or its relation with any antimicrobial peptide produced by bacteria was never been

assigned. The predicted molecular weight of the total protein (7225.42 Da) did not match with the observed molecular weights (5372.6 and 5502.5 Da) (Figure 4). In addition, MALDI-TOF of the BS1 active peptide allows ascertaining the molecular mass predicted by the correspondent SDS-PAGE with higher reliability. One possible explanation which can be speculated is the fact that our Bs1 peptide is a result of peptide maturation where the N-terminal extension of precursor peptide is cleaved off by the action of a certain enzyme that possible cleaves at a Met residue when the adjacent residues are non-bulky amino acids (Giglione *et al*., 2004).

3.6.2. N-terminal amino acid sequencing and analysis

Bs1 was subjected to automated Edman degradation to determine their N-terminal amino acid sequence. Their analysis provided the identification of a partial sequence A(N,V,I,L)-A(Q,P,I)-?(P,A,R,Q,D)-R(L,F,G,H)-?-I(T,Q,M,G)-L(Q)-Q(K)-K-L(V)-V-T(V,S)-S(T)-?-K(F), which present some uncertain positions in the assigned amino acids in brackets. However, a database search revealed homology with the same hypothetical protein as determined by MS/MS approach, namely gi 489307730 from *B. pumilus* ATCC 7061^{T}.

Therefore, custom synthesis was performed and synthetic peptide (MLDRFLNKMTNVQPAASRCILQKLVTSCKSAPTCFLQGVGTFRYYVDRDTGEICTSQERIRCGC) was evaluated on its antibacterial activity against *S. aureus*. Results evidenced that linear structure of this peptide have not any effect in the growth of *S. aureus*. The absence of cationic amino acids in the N-terminal sequence of this 64 amino acid peptide, reported as a crucial factor for antimicrobial activity, could be a possible justification for this result.

In addition, comparison of resulting N-terminal sequence and peptide gi 489307730 seems to suggest a N-terminal staggered from position 11 or 15. In this sense, further partial custom syntheses of the partial sequence SRCILQKLVTSCKSAPTCFLQGVGTFRYYVDRDTGEICTSQERIRCGC should be performed and antibacterial activity tested.

3.6.3. Secondary structure prediction

Circular dichroism (CD) spectroscopy was employed in an attempt to elucidate secondary structure integrity of the 5kDa purified peptide. The linear conformation of the synthesized synthetic peptide was used for comparison. CD spectra suggested that Bs1 peptide presented a random coil, e.g. unstructured nature in solution with a characteristic minimum at approximately 200 nm (Figure 6 and 7).

Most naturally antimicrobial peptides occurring in nature are cationic (i.e., the net charge at neutral pH varies from + 2 to + 9) and amphipathic molecules, which enables the peptides to interact with and disrupt lipid membranes. Moreover, they are very short in length, containing 5 to 40 amino acid residues, while others containing more than 40 residues.

Amino acid sequences of different AMPs are highly heterogeneous, nevertheless, positively charged residues such as Lys and Arg and substantial hydrophobic residues (~ 30% or more) are commonly found in these peptides class. In addition, a great variation in their secondary structures has also been reported, such as α-helices, β-sheets, or extended polyproline-like helices, due to the amphipathicity of these molecules which enhanced their antimicrobial mechanism of action.

Nevertheless, most of the linear AMPs are unstructured in solution, with some exceptions, e.g. LL37, a human antimicrobial peptide (Wildman *et al*., 2003; Dürr *et al*., 2006) and cyclic peptides due to the presence of one or more Cys–Cys disulfide bonds which possibly the formation β-sheets (Dhople *et al*., 2006).

Moreover, these unstructured (linear) cationic peptides present the possibility to embrace an amphipathic α-helical structure. In fact, while these peptides lack a secondary structure in solution, interactions with lipid bilayers in membranes induce an amphipathic α-helical structure that is believed to be a pre-requisite for its antimicrobial activity (Jin et al 2005) by the formation of pore-like structures (He *et al*., 1995; Matsuzaki *et al*., 1995) or a more general disruption (Oren *et al*., 1998).

Therefore, and since the mechanism by which an AMP executes its function depends on a number of physicochemical properties, such as the amino acid sequence, net charge, amphipathicity, hydrophobicity, structural folding (includes secondary structure, dynamics and orientation) in membranes, oligomerization, peptide concentration, and membrane

composition (Shai, 1999), further studies should be conducted in order to clarify the mechanisms of action of the unstructured Bs1 AMP to explain its antimicrobial activity.

3.6.4. Detection of possible post-translational modifications: glycosylation

Using SDS–PAGE electrophoresis followed by PAS staining, we did not observe any positive reaction for the purified BS1 CFS (Figure 8 (a)). Nevertheless, since limitations of this method are known, namely in the nature and in the degree of protein glycosylation, this results and evaluation of other possible post-translational modifications, as the determination of the presence of lipid moieties, should be attempted.

3.6.5. Prediction of protein antimicrobial regions

Due to the growing interest in AMPs, web applications as AMPA, based on algorithms, which provides the prediction of antimicrobial domains in proteins and can be used to propose short AMP, have been developed (Lata *et al.*, 2007; Fjell *et al.*, 2009; Torrent *et al.*, 2011; Wang *et al.*, 2011). This platform uses an antimicrobial propensity scale to generate an antimicrobial profile, for which antimicrobial IC50 values for all amino acid are determined. Therefore, from the IC50 data, an antimicrobial index is calculated, providing a tendency of such amino acid to be found within an AMPs sequence.

Considering Bs1 sequence presenting N-terminal sequence starting in amino acid 15 from gi 489307730, SRCILQKLVTSCKSAPTCFLQGVGTFRYYVDRDTGEICTSQERIRCGC, AMPA detect an antimicrobial domain, presenting a high probability to pertain to an antimicrobial peptide (Figure 9). This antimicrobial stretch was found in amino acids 1 to 13 positions, SRCILQKLVTSCK, from the resulting peptide. Statistic results revealed a propensity value 0.226 (9 %) and a mean antimicrobial value of 0.243, which permit to infer with 91% of probability that this sequence pertain to an antimicrobial peptide, supported by a low antimicrobial index, which also favor to be part of an AMP.

In fact, an adequate combination of hydrophobic and cationic amino acid residues, such as Arg, Lys, Gly, Cys, Trp and Val, underlies the action of antimicrobial regions, generally by conferring them an amphipathic secondary structure that favors interaction with microbial membranes, which can induces cell damage (Wimley, 2010). Therefore, the antimicrobial activity is usually confined to discrete stretches of the protein, often located

at the N- or C-terminal of proteins, rather than spread over the entire sequence (Ramanathan *et al*., 2002). When gi 489307730 was analyzed by AMPA the stretch RCILQKLVTSCK is assigned to pertain to an antimicrobial peptide, with a propensity value 0.229 (12 %) and a mean antimicrobial value of 0.246. This results can confirm our previously assumption, that N-terminal of antimicrobial mature protein derived from gi 489307730 start in position 17.

Moreover Collection of Antimicrobial Peptide (CAMP) database (http://www.camp.bicnirrh.res.in/) was also used to confirm the previous established antimicrobial region, using different statistical algorithms, such as Support Vector Machine (SVM), Random Forests (RF) and Artificial Neural Network (ANN) (Schaffer *et al*., 2001). For biological activity structural or physical parameters could be considered critical, namely size, sequence, charge, degree of structuring (helicity), hydrophobicity, amphipathicity and angles subtended by hydrophobic and hydrophilic faces of the formed helix (Faccone *et al*., 2014). Therefore, physicochemical properties as amino acid composition, net charge, hydrophobicity, aliphatic index, instability index and pI were determined and results summarized in table 2.

Physicochemical properties of the 48 amino acids predicted antibacterial peptide, revealed a pI of 8.65, an aliphatic index of 68.96, a net charge of 3 (total number of negatively charged residues (Asp + Glu): 4; total number of positively charged residues (Arg + Lys): 7), a hydropathy of -0.15 and an instability index of 46.36 (which classifying the peptide as unstable are in agreement with CD data).

Moreover, using antimicrobial peptides and proteins collections deposited in CAMP database, homologous sequences searches using BLAST were performed (Table S1). Nevertheless, no significant similarity was observed among our antimicrobial peptide with available AMP´s present in this database, suggesting that probably a new peptide can be involved in the reported activity, despite some reported antimicrobial proteins and peptides, including a short fraction of subtilin, present some similarity with our peptide.

Therefore, our finding and predictions prompt us to suggest the presence of a new alternative antibiotic as a potential candidate to circumvent *S. aureus* infections and thus a good possibility for its inclusion in topical use. Further investigations should be carried out to confirm the proposed amino acid composition and its structure elucidation. Moreover, its antimicrobial activity against a panel of clinical isolates expressing different mechanisms of resistance should also be extended.

Table 1. Strains used in antimicrobial activity assays.

Isolates	Origin	Susceptibility profile	Reference
Gram positive			
Staphylococcus aureus ATCC 29213		NA	ATCC
Staphylococcus aureus MRSA FFUP 001		Methicillin resistant	FFUPCC
Staphylococcus epidermidis Ste1	Catheter - Portugal (2012)	Methicillin- and Linezolid- resistant	Barros *et al*., 2014
Staphylococcus epidermidis 55	Human commensal flora– Italy (2011)	Susceptible	Cavallo, 2011
Enterococcus faecium (VRE) E1	Hospital sewage – Portugal (2001)	Vancomycin resistant	Freitas *et al*., 2009
Bacillus subtilis BGA		NA	Spore suspension, Merck (Product number 1106490001)
Bacillus pumilus ATCC 14884	Animal commensal flora– Portugal (2004)	NA	ATCC
Bacillus safensis Bs1		Intermediate profile to Cefotaxime	Branquinho *et al*., 2014
Bacillus cereus FFUP 95		NA	FFUPCC
Lysinibacillus fusiformis	Animal commensal flora– Portugal (2004)	Intermediate profile to Clindamycin	FFUPCC
Listeria monocytogenes FFUP 35		NA	FFUPCC
Gram negative			
Acinetobacter baumanni ATCC 19606		NA	ATCC
Escherichia coli ATCC 25922		NA	ATCC
Pseudomonas aeruginosa ATCC 27853		NA	ATCC
Salmonella Typhymurium F154/22	Food – Portugal (2004)	Nitrofurantoin resistant	FFUPCC; Antunes *et al*., 2006
Salmonella Enteritidis 62/02	Human – Portugal (2002)	Susceptible	INSA; Antunes *et al*., 2006

NA – not applied; ATCC - American Type Culture Collection; FFUPCC - Faculty of Pharmacy of Porto University Culture Collection; INSA - National Institute Dr. Ricardo Jorge Culture Collection

Table 2. Prediction of antimicrobial regions accordingly CAMP database.

Algorithm	Class	AMP Probability
Artificial Neural Network (ANN) classifier	AMP	NA
Discriminant Analysis classifier		0.884
Random Forest Classifier		0.7465
Support Vector Machine (SVM)		0.646

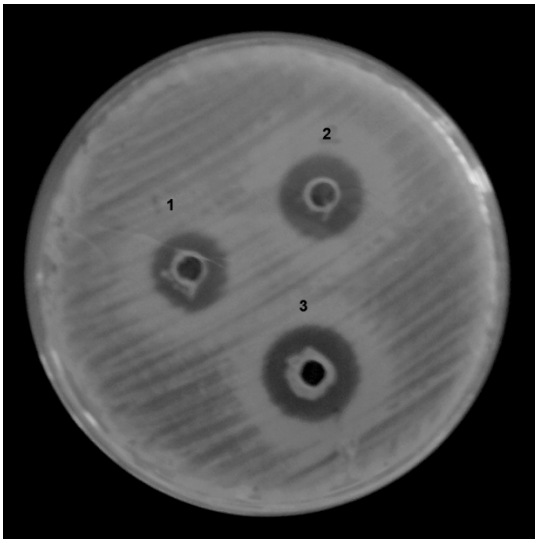


Figure 1. Antibacterial activity against *S. aureus* ATCC 29213 of the purified peptide recovered from *B. safensis* Bs1, using well diffusion assay. Legend: (1) direct Bs1 CFS, (2) ultrafiltered fraction followed by peptide precipitation using 40% (w/v) and (3) 60% (w/v) of ammonium sulfate saturation. Bs1 peptide revealed 14, 16 and 17 mm of inhibition zones for *S. aureus* ATCC 29213, respectively. Similar results were obtained in the three individual experiments.

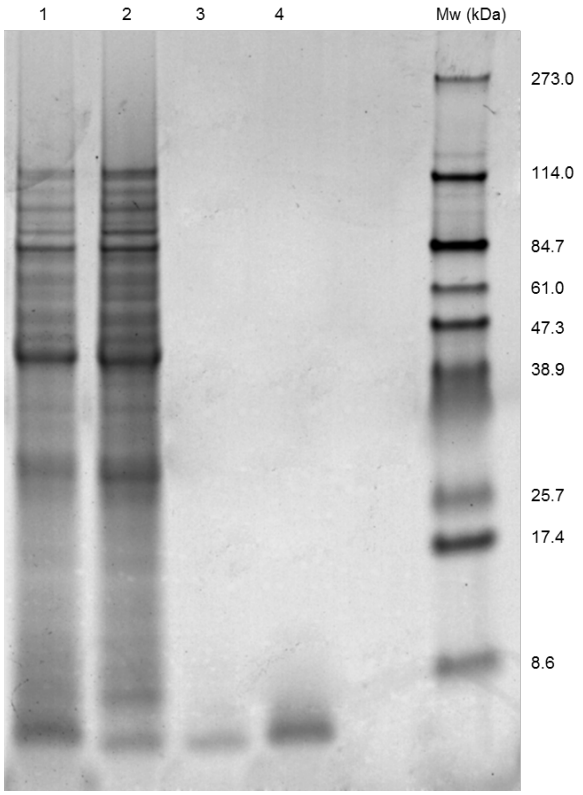


Figure 2. SDS-PAGE of the precipitated proteins from CFS of *B. safensis* Bs1. Lane 1: direct CFS Bs1, Lane 2: fraction ultrafiltered throughout >10 kDa, Lane 3: fraction ultrafiltered followed by peptide(s) precipitation using 40% (w/v) ammonium sulfate saturation, Lane 4: fraction ultrafiltered followed by peptide(s) precipitation using 60% (w/v) ammonium sulfate saturation, Mw: Molecular weight marker.

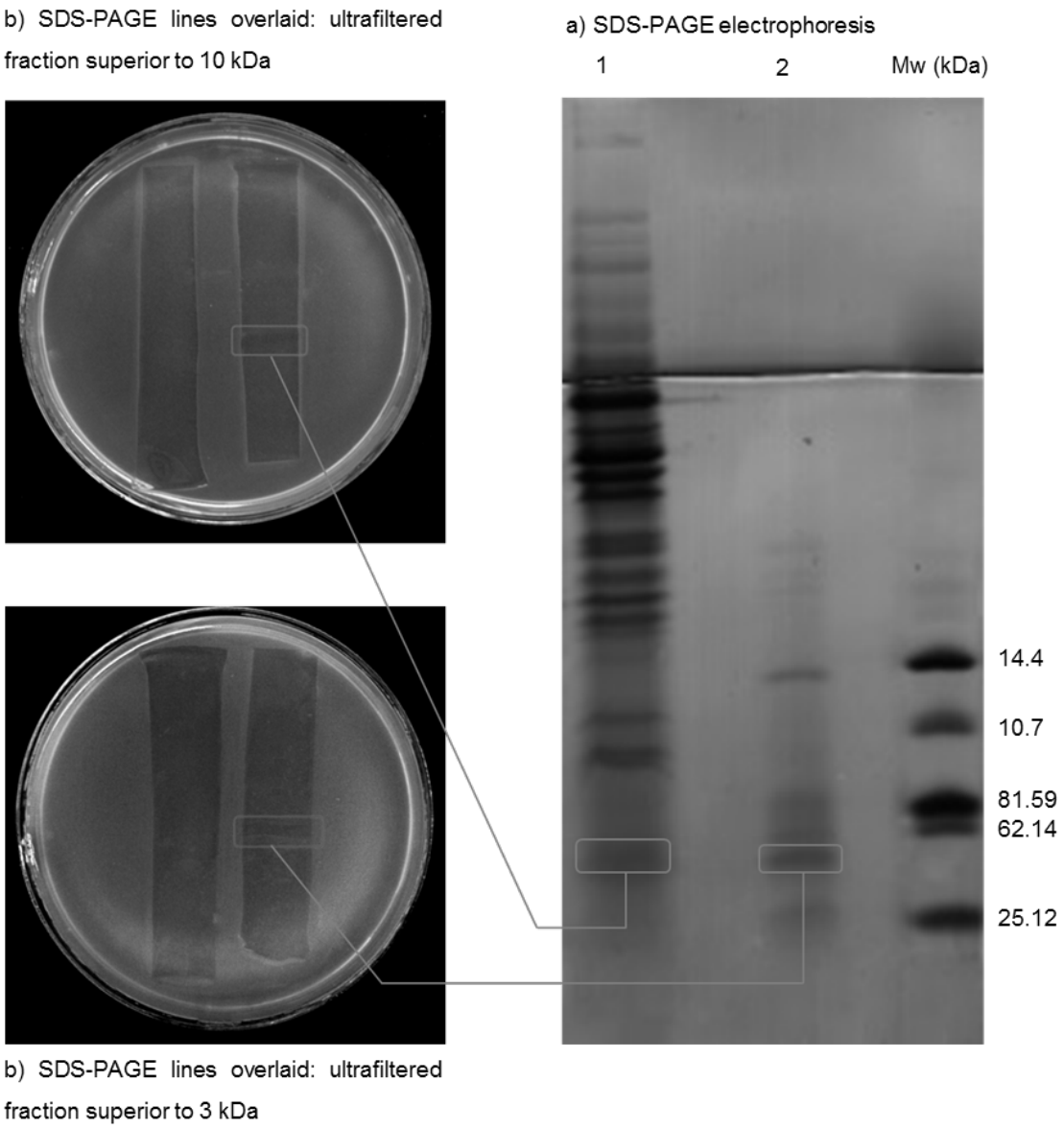


Figure 3. SDS-PAGE of the ultrafiltered peptide(s) recovered from CFS of *B. safensis* Bs1 (a). Lane 1: CFS Bs1 ultrafiltered throughout >10 kDa, Lane 2: CFS Bs1 ultrafiltered throughout >3 kDa, Mw: Molecular weight marker. (b) and (c) direct detection of antimicrobial activity by overlaying the gel with nutrient agar containing the indicator strains *S. aureus* ATCC 29213 of the ultrafiltered fraction >10 kDa da and > 3 kDa, respectively . Inhibition zones were observed after 24h of incubation at 37ºC.

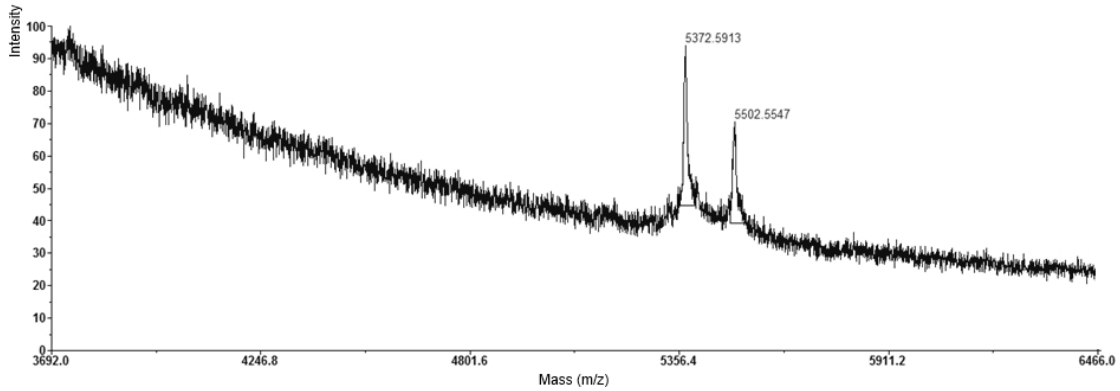


Figure 4. MALDI-TOF/MS of Bs1 peptide(s) acquired in positive linear mode from the resolved SDS-PAGE gel band purified using 60% (w/v) of ammonium sulfate saturation. A similar spectrum was obtained for 40% (w/v) of ammonium sulfate saturation.

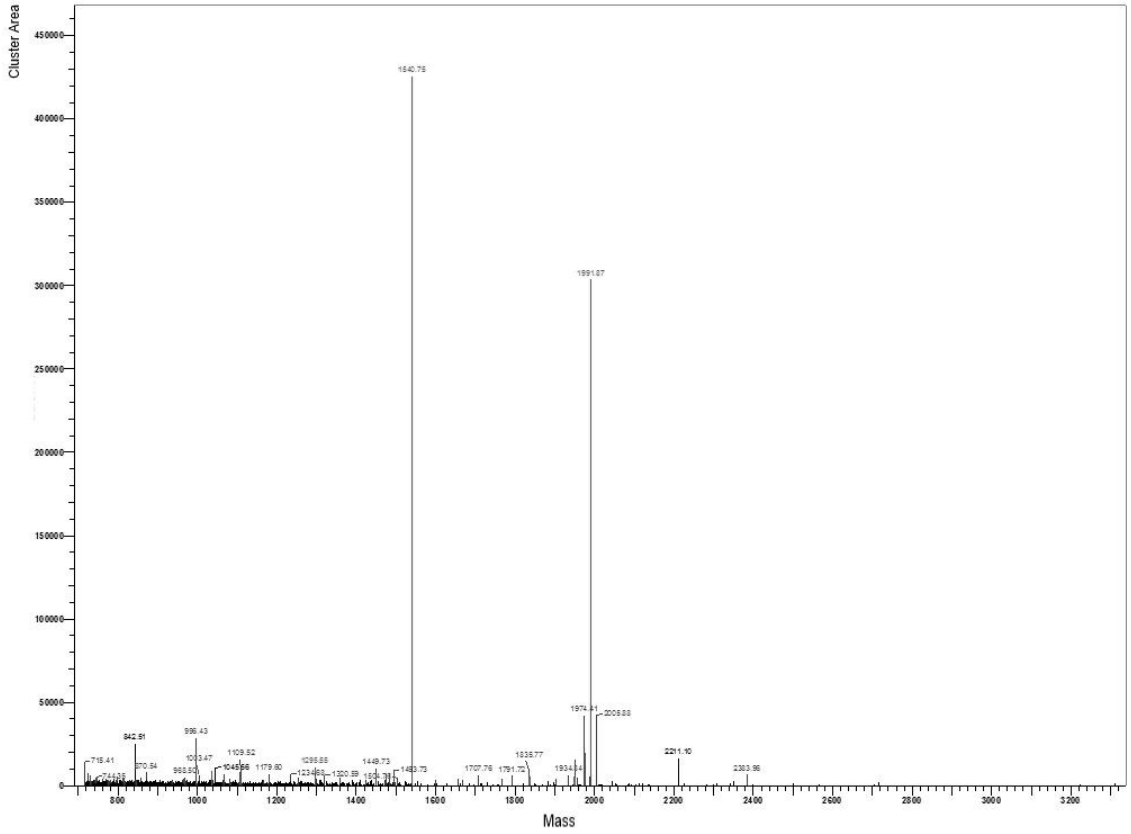


Figure 5. PMF analysis of Bs1 antimicrobial peptide. PMF followed by MASCOT search shows the matched residues (in red) of the peptide fragments with the putative protein (gi 489307730) in genome of *B. pumilus* ATCC 7061^{T} with 100% of identity.

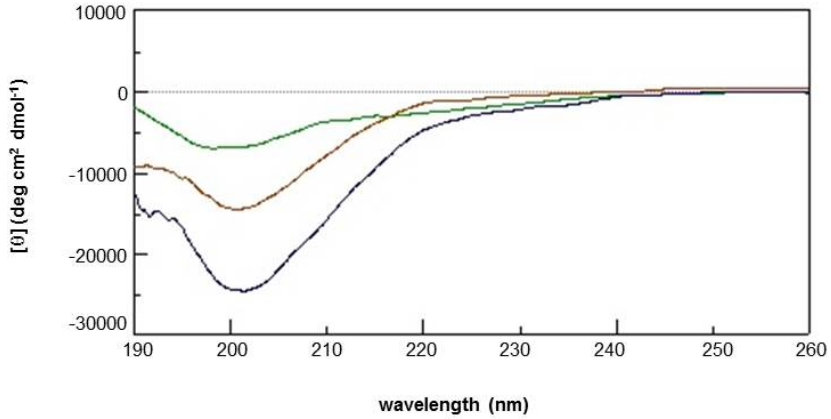
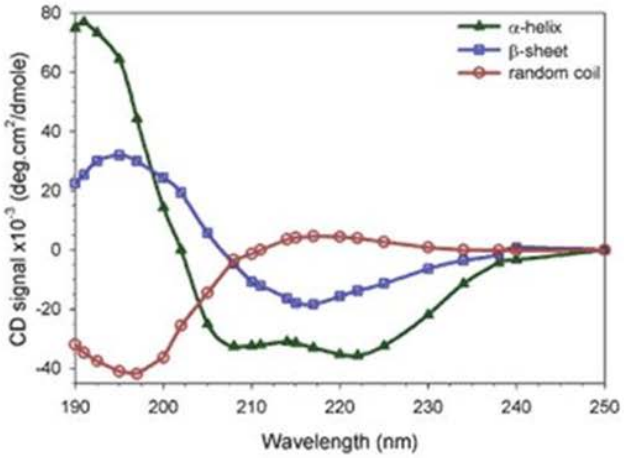
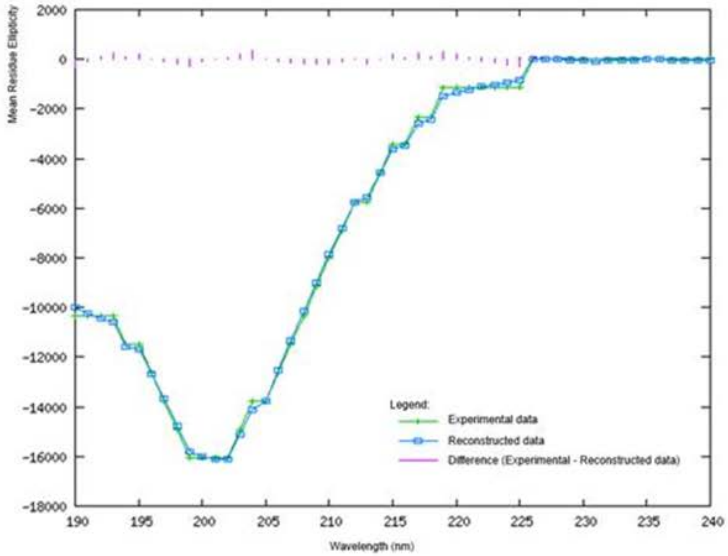


Figure 6. Circular dichroism spectra, recorded in the far-UV region (190–260 nm), of purified Bs1 antimicrobial peptide using precipitation with 40% (w/v) of ammonium sulfate saturation followed by C18 ZipTips preparations (blue), CFS ultrafiltered fraction (>3000Da) (red) and synthetic Bs1 peptide (green), revealing its intrinsically disordered nature in solution.

a) Representation of standard curves of the three basic secondary structures of a polypeptide chain (helix, sheet, coil) by Greenfield and Fasman (1969).



b) Dicroweb results derived from Bs1 peptide CD spectrum.



Secondary structure prediction accordingly CDSSTR method, using 7 reference databases in Dicroweb website.

Helix 1	Helix 2	Strand 1	Strand 2	Turns	**Unordered**	Total
0.01	0.03	0.16	0.08	0.13	**0.58**	0.99

Figure 7. Prediction of the protein secondary structure content estimated using Greenfield and Fasman method (a) and the DichroWeb server (b), supporting the unordered nature of Bs1 peptide in solution.

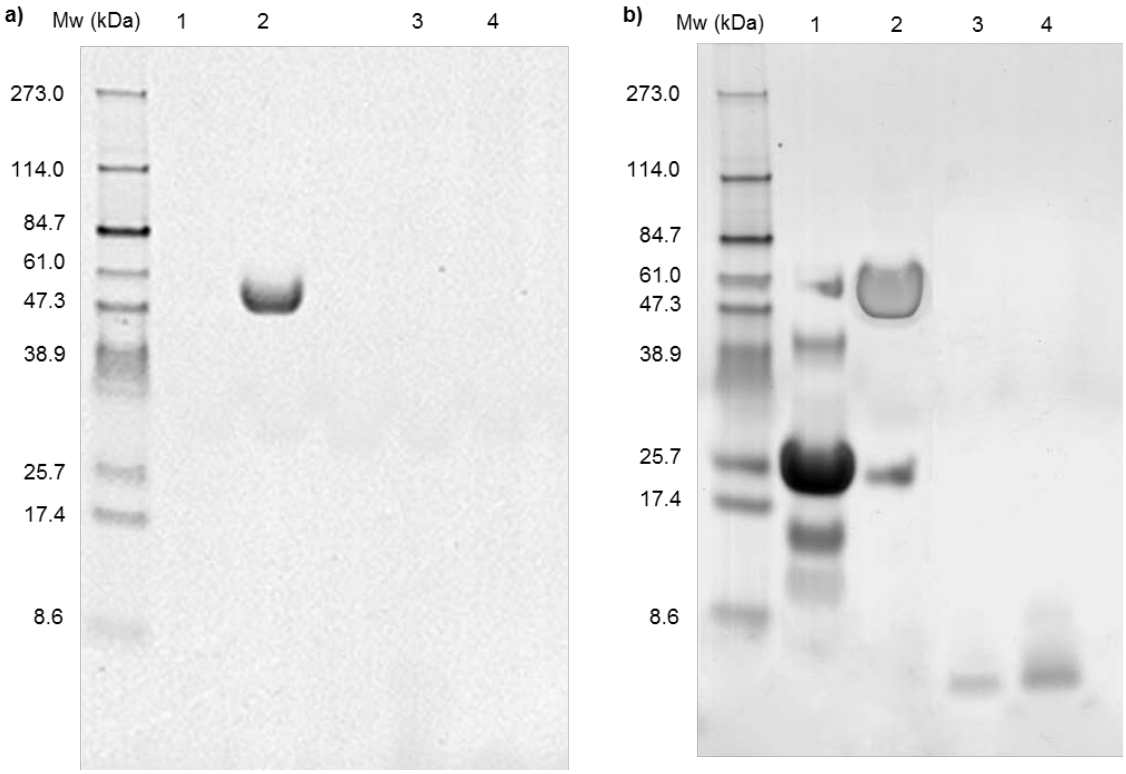


Figure 8. SDS-PAGE electrophoresis followed by PAS staining of the purified peptide recovered from CFS of *B. safensis* Bs1 (a). Lane 1: STI protein (negative control), Lane 2: HRP protein (positive control), Lane 3: fraction ultrafiltered followed by peptide precipitation using 60% (w/v) ammonium sulfate saturation, Lane 4: fraction ultrafiltered followed by peptide precipitation using 40% (w/v) ammonium sulfate saturation, Mw: Molecular weight marker. The same protein samples were electrophoresed in identical polyacrylamide gels and then stained with Comassie Blue to confirm the presence of the purified Bs1 peptide (b).

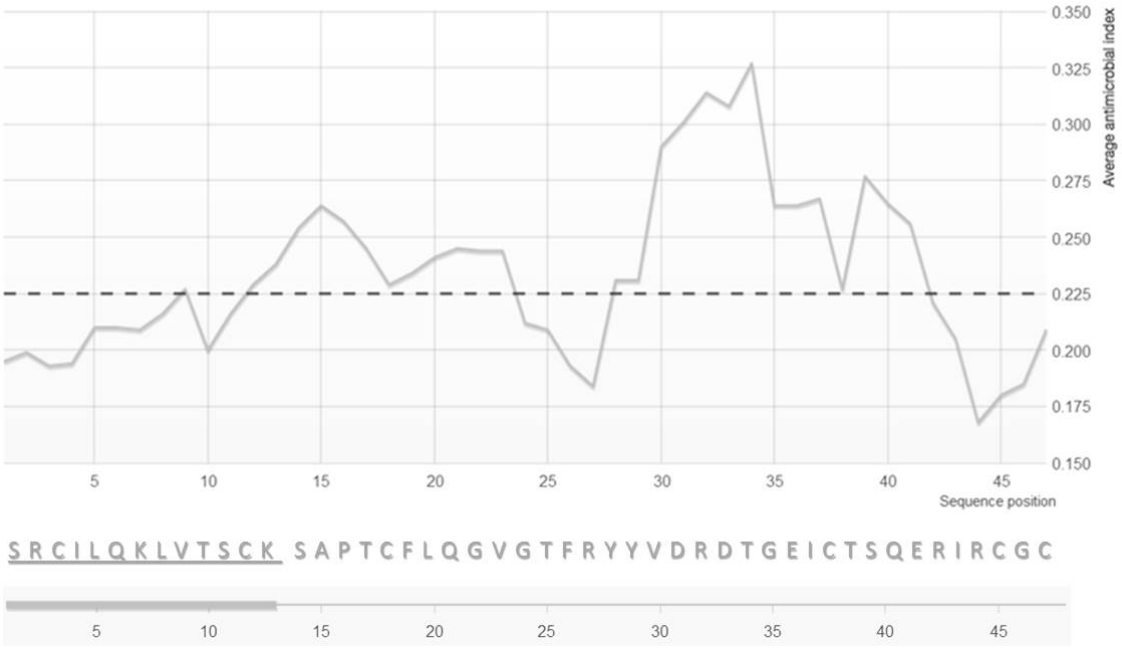


Figure 9. AMPA results from the antimicrobial chart profile of the Bs1 sequence peptide. On X-axis is reported the amino acid position in the peptide and on the Y-axis the antimicrobial score at that position. The probability values displayed correspond to the probability to find the predicted stretch in a non-antimicrobial protein.

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Supplementary data

Characterization of a new antimicrobial peptide with anti-MRSA activity produced by *Bacillus safensis* Bs1

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Running title: Antimicrobial peptides produced by *Bacillus safensis* Bs1

Table S1. Comparison resulting sequences producing significant alignment and blast results from homologous sequences using BLAST tool inside the CAMP database.

CAMP ID	UniProt ID	Protein name		Taxonomy	Sequence	Blast	Score (bits) Values	Expect	Identities
Bacteria									
CAMPSQ1204	P10946	Lantibiotic subtilin		*Bacillus subtilis*	WKSESLCTPGCVTGALQTCFLQTLTCNCKISK	Bs1 3 CILQKLVTSCK 13 C LQ L +CK Subtilin 19 CFLQTLTCNCK 29	18.1 bits (35)	9.9	6/11 (55%)
CAMPSQ3288	P36961	Lactococcin G-a		*Lactococcus lactis* LMGT2081	GTWDDIGQGIGRVAYWVGKALGNLSDVNQASRINRKKKH	Bs1 21 QGVGTFRYYVDRDTGEI 37 QG+G Y+V + G + Lactocin 8 QGIGRVAYWVGKALGNL 24	18.5 bits (36)	8.1	6/17 (35%)
Animalia									
CAMPSQ2390	E1B2T7	Brevinin [a)]	2LF2	Amphibians	IMSTLKQFGISAIKGAAQNVLGVLSCKIAKTC	Bs1 9 VTSCKSAPTC 18 V SCK A TC Brevinin LF2 23 VLSCKIAKTC 32	18.5 bits (36)	6.8	7/10 (70%)
CAMPSQ4290	K7ZGR3	Brevinin 2 [a)]	AJ1		GLMSTFKRVGISAIKGAAKNVLDVLSCKIAKTC	Bs1 9 VTSCKSAPTC 18 V SCK A TC Brevinin 2AJ1 24 VLSCKIAKTC 33	18.5 bits (36)	7.0	7/10 (70%)
CAMPSQ4284	K7ZAL2		AJ2		GLMSTFKQVGISAIKGAAKNVLDVLSCKIAKTC	Bs1 9 VTSCKSAPTC 18 V SCK A TC Brevinin 2AJ2 24 VLSCKIAKTC 33	18.5 bits (36)	7.0	7/10 (70%)
CAMPSQ4289	K7Z432		AJ3		GLMSTFKQVGISAIKGAAQNVLGVLSCKLAKTC	Bs1 9 VTSCKSAPTC 18 V SCK A TC Brevinin 2AJ3 24 VLSCKLAKTC 33	18.5 bits (36)	7.0	7/10 (70%)
CAMPSQ4287	K7ZAL3		AJ7		GLVSTFKQVGISAIKGAAKNVLDVLSCKIAKTC	Bs1 9 VTSCKSAPTC 18 V SCK A TC Brevinin 2AJ7 24 VLSCKIAKTC 33	18.5 bits (36)	7.0	7/10 (70%)
CAMPSQ4283	K7ZJ56		AJ4		GFMSTFKQVGISAIKGAAKNVLDVLSCKIAKTC	Bs1 9 VTSCKSAPTC 18 V SCK A TC Brevinin 2AJ4 24 VLSCKIAKTC 33	18.5 bits (36)	7.0	7/10 (70%)
CAMPSQ1301	Q9U6U0	Antimicrobial peptide MGD2b		Molluscs (Bivalvia)	MKAVFVLLVVGLCIMMMDVATAGFGCPNNYACHQHCKSIRGY CGGYCASWFRLRCTCYRCGGRRDDVEDIFDIYDNVAVERF	Bs1 35 GEICTSQERIRCGC 48 G C S R+RC C MGD2b 44 GGYCASWFRLRCTC 57	19.6 bits (39)	4.7	7/14 (50%)

[a)] antimicrobial peptide precursor

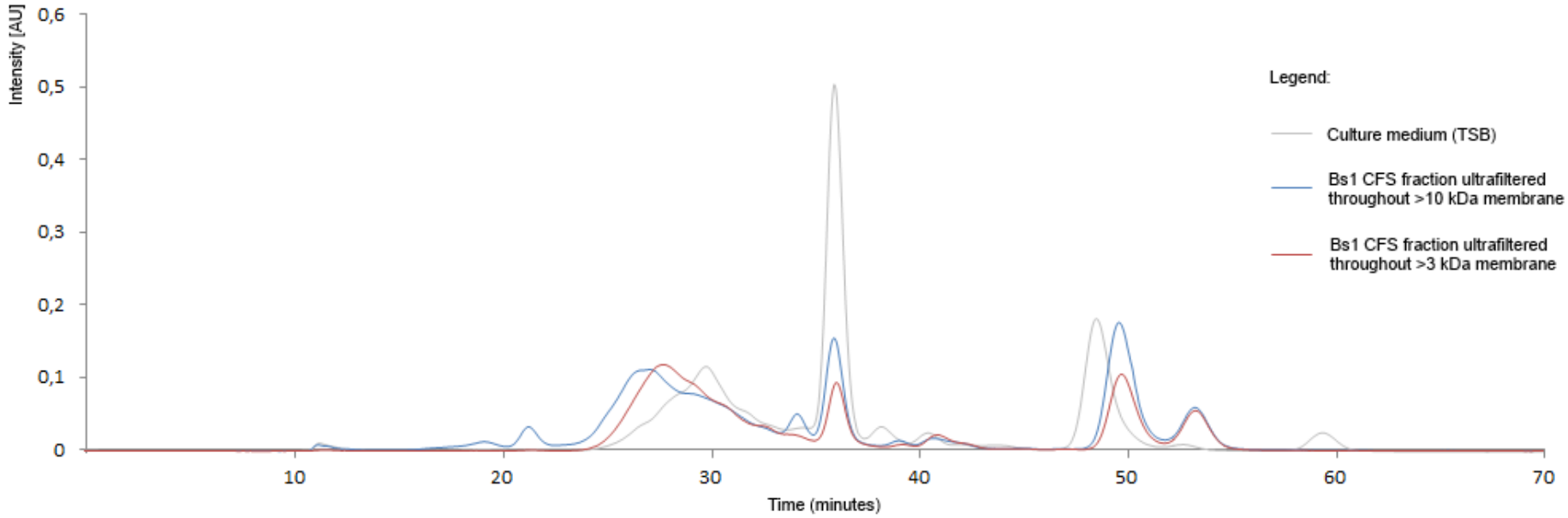


Figure S1. (a) FPLC chromatogram profiles of CFS Bs1 peptide(s) pre-purified using ultrafiltration throughout >10 kDa and >3kDa membranes, demonstrating the presence of an additional peak (when compared with the negative control - TSB), in the retention time between 50 and 60 min, which possibly corresponds to the active peptide with 5 kDa.

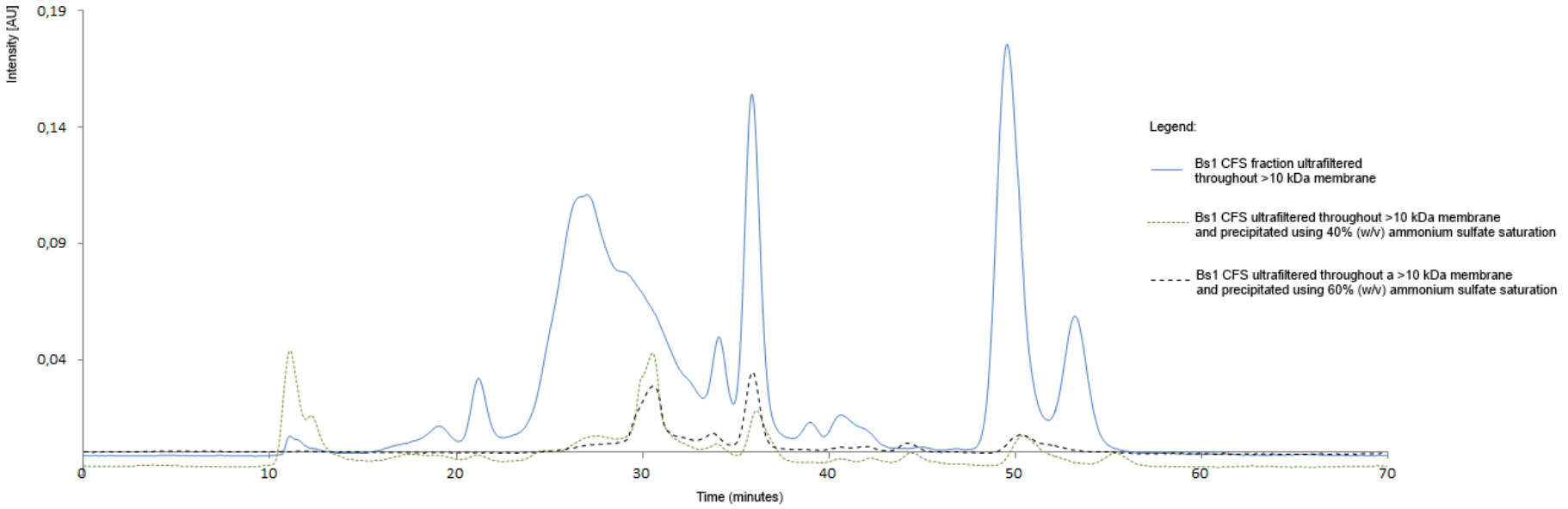


Figure S1. (b) FPLC chromatogram profiles of CFS Bs1 peptide(s) pre-purified using ultrafiltration throughout >10 kDa followed by and ammonium sulfate precipitation (40% and 60% of saturation).

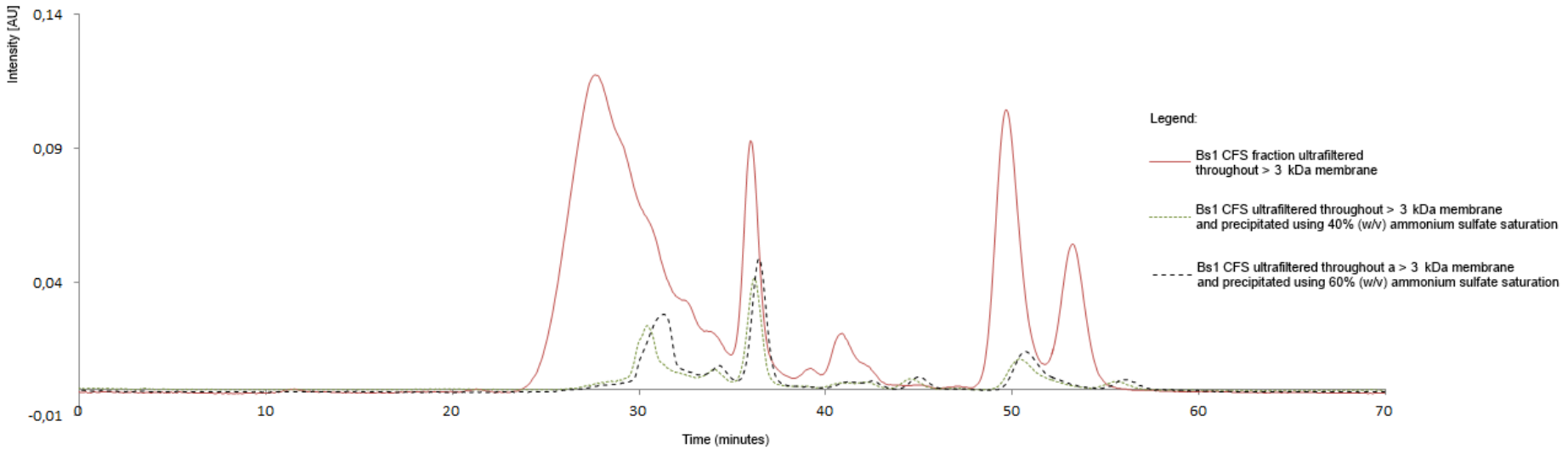
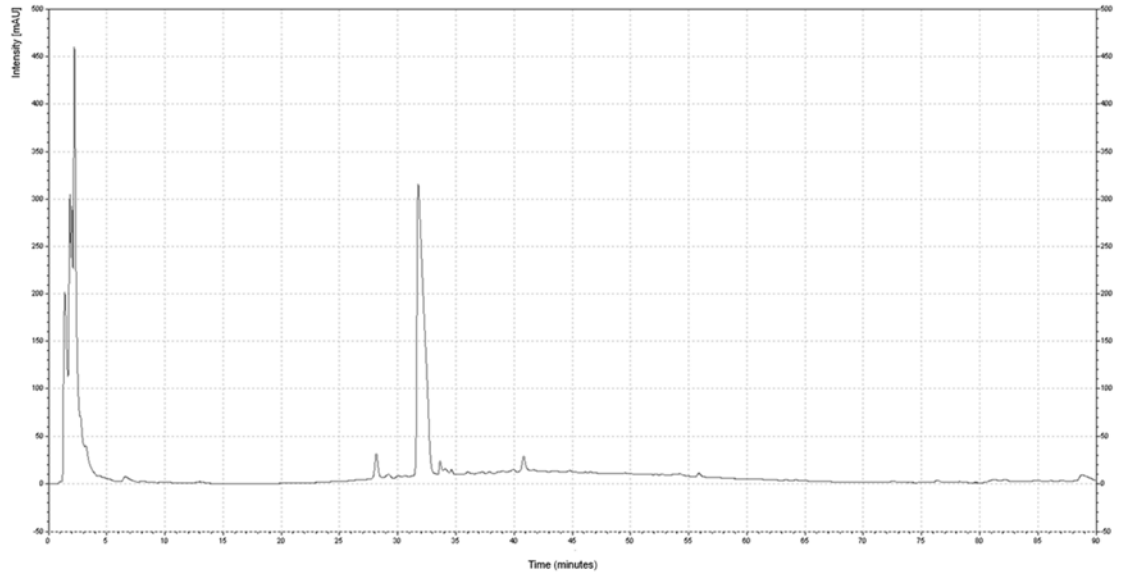
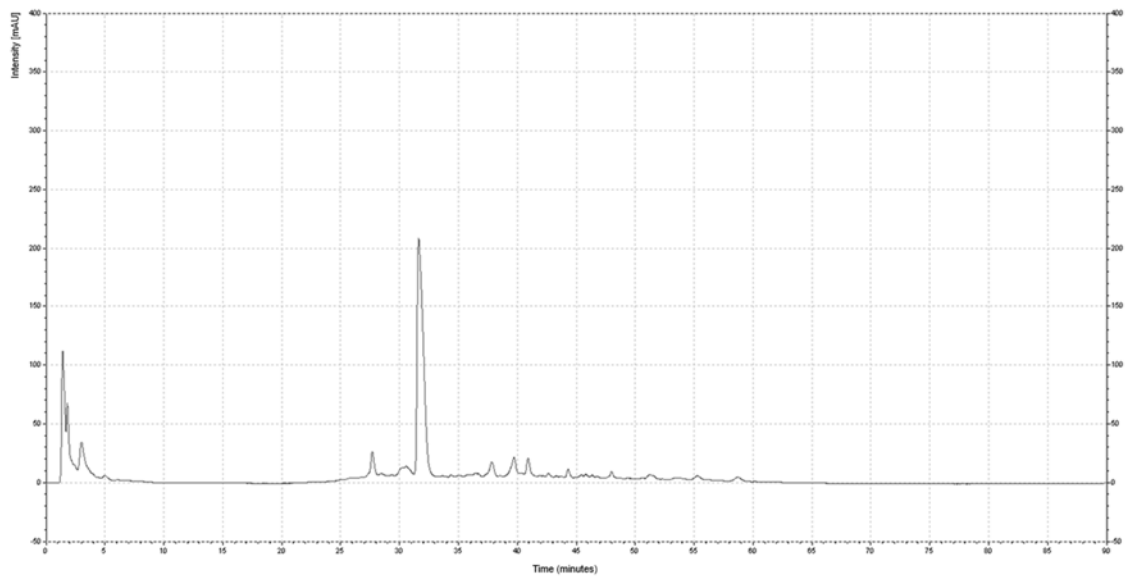


Figure S1. (c) FPLC chromatogram profiles of CFS Bs1 peptide(s) pre-purified using ultrafiltration throughout >3 kDa followed by and ammonium sulfate precipitation (40% and 60% of saturation).

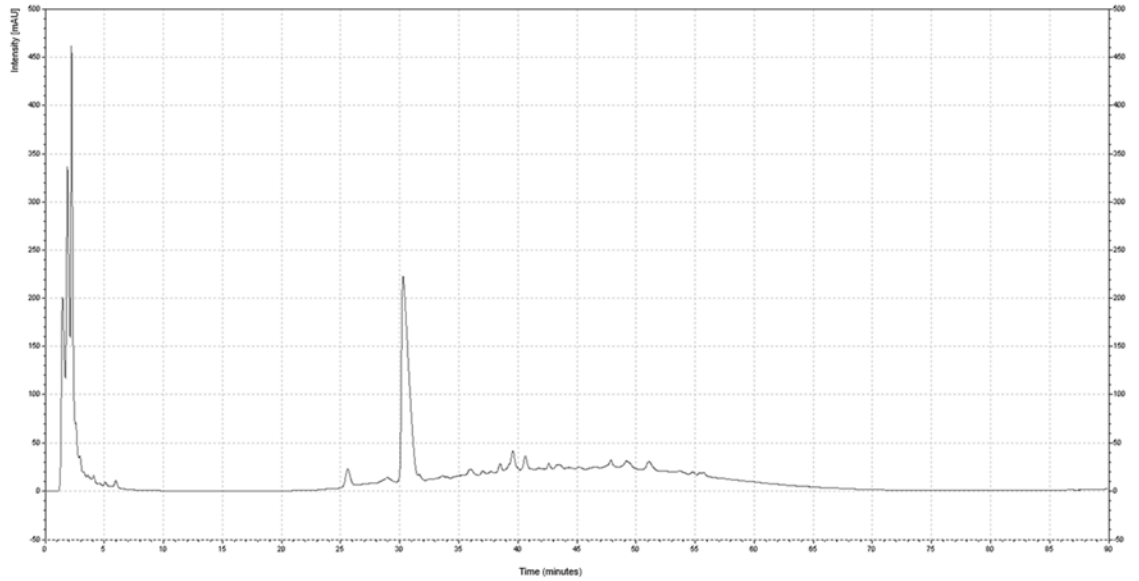
a) Culture medium (TSB)



b) Bs1 CFS fraction ultrafiltered throughout >10 kDa membrane



c) Bs1 CFS fraction ultrafiltered throughout >3 kDa membrane



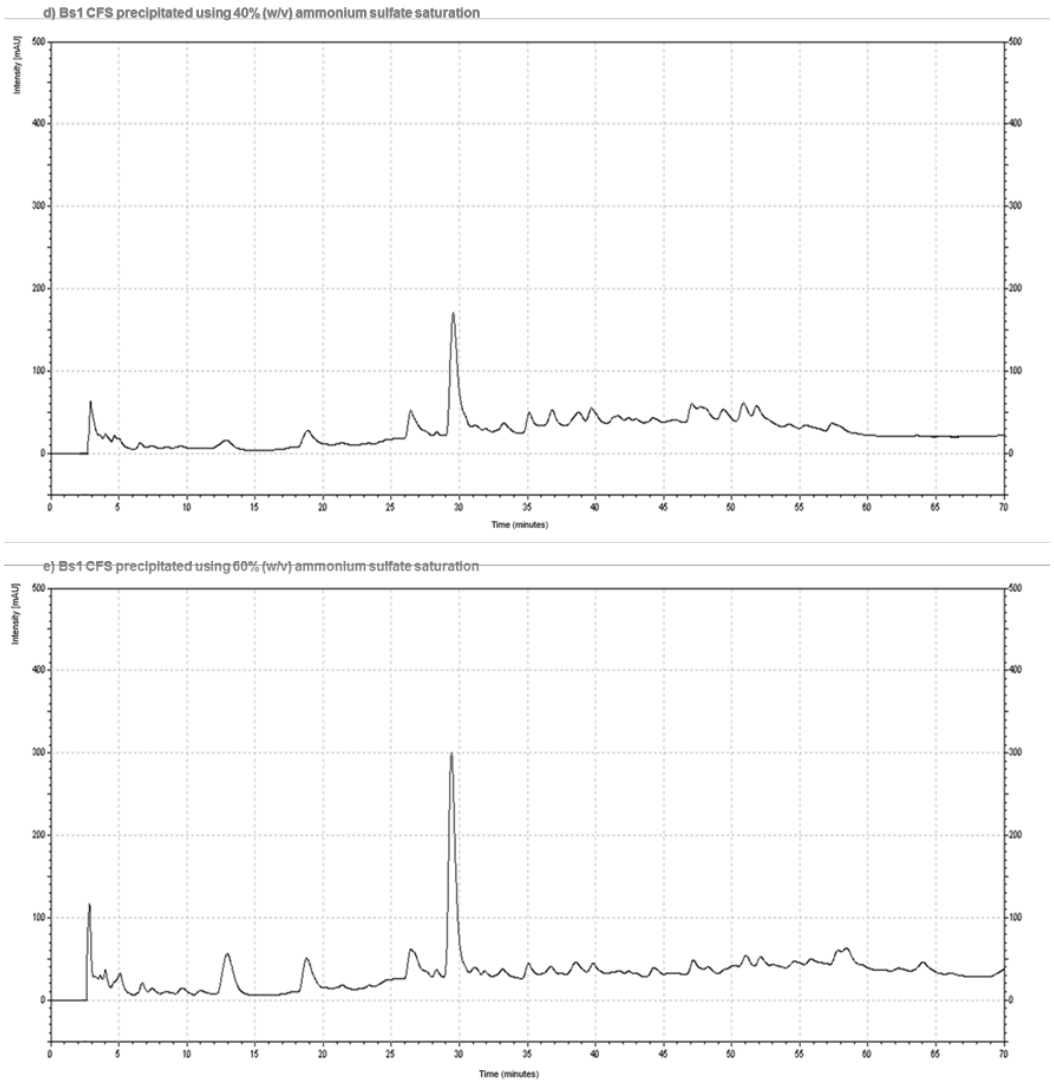


Figure S2. Reverse-phase HPLC chromatogram profiles of CFS Bs1 peptide (a) culture medium, (b) Bs1 CFS fraction ultrafiltered throughout >10 kDa membrane, (c) Bs1 CFS fraction ultrafiltered throughout >3 kDa membrane, (d) Bs1 CFS precipitated using 40% (w/v) ammonium sulfate saturation and (e) Bs1 CFS precipitated using 60% (w/v) ammonium sulfate saturation. Different fractions were lyophilized before the antibacterial assay.

CHAPTER 4

Biosurfactants in *B. pumilus* group species: diversity and anti-biofilm activity of a surfactin-like biosurfactant

4.1. Species-specific surfactin-like biosurfactants combination within *Bacillus pumilus* group

Publication:

Species-specific surfactin-like combinations of biosurfactants within *Bacillus pumilus* group

This work addresses the species-specific combinations of biosurfactants, namely surfactin-like compounds achieved in *Bacillus safensis* and *Bacillus pumilus*. Remarkably, *B. safensis* isolates revealed the production of a mixture of biosurfactants composed of pumilacidin variants and, in smaller amounts of a surfactin variant, whereas in *B. pumilus* only pumilacidin variants were detected. Moreover *B. altitudinis* isolates doesn´t revealed surface-active compounds production. In addition absence of correlation between detection of lipopeptides synthetase genes by a PCR approach and biosurfactant production was also detected.

Results disclosed from this work highlight these species as further exploring sources of bioactive compounds, broadening their potential applications.

Species-specific surfactin-like combinations of biosurfactants within *Bacillus pumilus* group

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Running title: Surfactin-like combinations within *Bacillus pumilus* group species biosurfactants.

Key-words: pumilacidin, surfactin, biosurfactants, *Bacillus pumilus*, *Bacillus safensis*

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Abstract

Assessing the diversity of biosurfactants produced by *B. pumilus* group species we verified surface-active properties compatible with biosurfactant compounds in most *B. pumilus* (n=3 of 4 isolates) and *B. safensis* (n=9 of 15) isolates tested, and their absence in *B. altitudinis* (n=9). Moreover, *srf/lch* (100%) and *fen* (52%) genes were frequently detected, although iturin family genes were not observed. Interestingly, LC/ESI-MS/MS analysis revealed a mixture of pumilacidin variants and surfactin compounds in *B. safensis*, whereas in *B. pumilus* only pumilacidin variants were detected. These data contrast with *B. subtilis* were surfactin and fengycin were detected.

In conclusion, despite the frequent detection of surfactin and fengycin genes in *B. pumilus* group isolates, surface-active properties were only associated with surfactin-like combinations in *B. safensis* and *B. pumilus*. Interestingly, a species-specific content in biosurfactant homologues was observed. Further studies of these biosurfactant variants could unveil interesting effects, broadening potential applications of these compounds.

1. Introduction

Biosurfactants (i. e. surface-active compound of microbial origin), are particular amphipathic molecules that recently have emerged as a promising class of bioactive secondary metabolites with increasing scientific, therapeutic and biotechnological interest (Banat et al., 2010, Fracchia et al., 2012, Gudiña et al., 2013, Martinotti et al., 2013, Seydlová et al., 2011).

Among the different current classes, lipopeptides, due to their structural novelty and versatility, represent one of the most important ones widely produced among *Bacillus* species (Fracchia et al., 2012, Kalinovskaya et al., 2002, Martinotti et al., 2013, Soberón-Chávez, 2011). Chemically, these compounds are constituted of 7–10 amino acids, which are cyclized by a lactone ring to a β-hydroxy fatty acid with distinct chain lengths. Moreover, they are classified into different families, encompassing surfactins (Arima et al., 1968), iturins (Delcambe et al., 1977), fengycins (Vanittanakom et al., 1986), bacillomycins (Roongsawang et al., 2002), mycosubtilin (Duitman et al., 1999) and kurstakins (Hathout et al., 2000).

Surfactin family encompasses about 20 different lipopeptides biosurfactants (also named surfactin-like compounds), which have been reported as widely produced by members of *Bacillus subtilis* complex (Soberón-Chávez, 2011). Moreover, *B. pumilus* species, taxonomically classified into *B. pumilus* group, which belong to *B. subtilis* complex, have also been described as surfactin-like producers, especially for a biosurfactant named pumilacidin (Melo et al., 2009, Morikawa et al., 1992, Naruse et al., 1990). Nevertheless, until our knowledge, its production was not demonstrated in other members of this group, namely in *B. safensis* and *B. altitudinis*.

Since the discovery of surfactin (Arima et al., 1968) and the characterization of its molecular structure as a macrolide lipopeptide, it has been recognized its remarkable surface-, interface- and membrane-active properties, which have resulted in a high number of promising biological activities of great relevance in biotechnology (Fracchia et al., 2012, Seydlová et al., 2011), medicine (Banat et al., 2010, Fracchia et al., 2012, Gudiña et al., 2013, Martinotti et al., 2013, Seydlová et al., 2011) and environmental protection (Pacwa-Płociniczak et al., 2011). Nevertheless, the knowledge concerning surfactin-like biosurfactants as a class of molecules remains limited. In fact, there's a lack of a concerted effort to perform a comprehensive screening for biosurfactants producers

and, in addition, the present knowledge in the surfactin-like biosurfactants has been developed around a relatively small number of well-characterized molecules.

Lipopeptide biosurfactants share a common mode of assembly through the action of multienzymatic proteins organized in modules, named non-ribosomal peptide synthetases (NRPSs) by a thiotemplate process (Tapi et al., 2010). Although the genetics of surfactin biosynthesis and its control have been most extensively studied in *B. subtilis* (Tapi et al., 2010), genes homologous to their peptide synthetases have not yet been studied in *B. pumilus* group. Nevertheless, molecular approaches solely might not be useful for the screening of biosurfactant producers (Soberón-Chávez, 2011) thus methodologies for the detection of putative biosurfactant-like activity, such as surface tension analysis, should be incorporated for accurate detection/screening of these compounds.

In this study we assessed the ability of species enclosed in *Bacillus pumilus* group to produce biosurfactants, screening for surface-active properties and representative lipopeptide family genes, in isolates collected from diverse sources and geographic locations. Moreover, structure and composition of the produced bioactive products was elucidated by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) analysis.

2. Material and Methods

2.1. Bacterial isolates

Fifteen *B. safensis*, nine *B. altitudinis* and four *B. pumilus* isolates previously identified by genotypic (16S rRNA, *gyrB and rpoB* gene sequences) methods were studied (Branquinho et al., 2014). They were recovered from different geographic terrestrial locations and sources, including food samples (Norway, Italy and Africa) (n=6), plants (USA) (n=4), gastropods (Portugal) (n=2), health (n=12) and cosmetic (n=4) products (Portugal). *B. pumilus* ATCC 14884 was also include in the analyses. Additionally, a *B. subtilis* isolate recovered from a health product contaminant was included for comparison (Table 1).

Bacillus isolates were maintained on Luria Bertani-Miller (LB) agar (Oxoid, United Kingdom) for short-term storage and in LB broth supplemented with 40% (v/v) glycerol at -80ºC for long-term storage.

2.2. Biosurfactant production and purification

For biosurfactant production, a seed culture was prepared for each isolate by transferring a loop from a LB agar overnight culture into 10 mL of LB broth incubated for 4 h, at 28°C and 200 rpm, on an orbital shaker. Thereafter, 2 ml of these cultures were inoculated into 500 mL of LB broth and incubated at 28°C and 120 rpm for 24 h. A cell-free preparation from these cultures was then prepared by centrifuging at 8,000 g^{-1} for 30 min. This supernatant was used for qualitative measurement of surface tension.

For biosurfactant extraction, the cell-free preparation was acidified to pH 2 with HCl (6 mol L^{-1}), and left for 12h at 4ºC for precipitation. The precipitate was then removed by centrifugation at 8,000xg for 10 min at 4ºC and the resulting solution extracted twice with ethyl acetate/methanol (4:1 v/v) mixture according to the method described by Rivardo et al. (2009). The remaining water present in the organic phase was then removed by anhydrous sodium sulfate. Finally, the organic phase was evaporated to dryness under vacuum condition and acetone was added to recover raw biosurfactant. Acetone was, then, evaporated and biosurfactant collected, weighted and quantified.

2.3. Evaluation of surface-active properties

2.3.1. Qualitative measurement of surface tension.

Screening of surface-active properties of biosurfactants was firstly conducted on the supernatant obtained as described in section 2.2, using the oil spreading assay (Morikawa et al 2000). Briefly, 20 µl of Motor Oil 10 W-40 (Selenia) were deposited onto a surface of 20 mL of distilled water to form a thin layer. Subsequently, 20µl of the *Bacillus* spp. supernatants were gently put onto the center of the oil layer and the diameter of the oil displacement circle formed was measured to determine the presence of biosurfactant.

2.3.2. Quantitative measurement of surface tension.

Surface active tension of all supernatants revealing a positive result in the oil spreading assay was determined using a Sigma 703D tensiometer (KSV) equipped with a Du Noy platinum ring at 35 ºC, on a 20 ml of a biosurfactant solution which was prepared in alkaline sterile demineralized water at 500 µg ml^{-1}. Distilled water was used for calibration. Critical Micelle Concentration (CMC) was then determined on serially diluted biosurfactant solutions in alkaline distilled water and was estimated from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections of a curve plotted between biosurfactant concentration and surface tension values (Rivardo et al., 2009)

2.3.3. Screening of bioemulsifier producers

The capacity of these biosurfactants to emulsify a hydrophobic substance was also tested. Cell-free supernatants (5 mL) obtained in section 2.2 were mixed with 5 mL of hexadecane with a vortex mixer and allowed to stand for 24 h, 8 days and 3 months at room temperature. After this time the emulsifying index was determined. E_{24} index is defined as percentage of height of emulsified layer (mm) divided by the total height of the liquid column (mm) (Iqbal et al., 1995).

2.4. Detection of lipopetide synthethase genes by PCR

The presence of genes *srf/lch, sfp, lchAA, lchAB, lchAC, fen, fenA*, *fenB*, *fenD*, *pps*, lpa-14 and ituD, encoding lipopeptides families (surfactins/lichenysins, iturins and fengycins/plipastatins) previously reported in *Bacillus* spp., was searched by PCR, using degenerated and specific primers, and sequencing (Table 1) (Athukorala et al., 2009, Hsieh et al., 2004 and 2008, Nieminen et al., 2007, Tapi et al., 2010). Sequences obtained were compared on GenBank database using the BLAST search.

2.5 Characterization of lipopeptides by LC/ESI-MS/MS

2.5.1 Mass spectrometry analysis

An aliquot of the biosurfactant extract was dissolved in methanol/acetonitrile (50/50 v/v) to obtain a 1000 µg ml^{-1} stock solution. Freshly prepared working solutions were made by diluting the stock solution with methanol/water (50/50 v/v) to achieve 15 µg ml^{-1} solutions.

All mass spectrometry analyses were done on a LCQ DECA XP Plus (Thermo Finnigan, San Jose, CA, USA), Ion Trap mass instrument equipped with an ESI source. Samples (15 µg ml^{-1} solutions) were infused with a syringe at 5µg min^{-1} flow rate. Source voltage and capillary voltage were 4.80 kV and 23 V in positive ion mode, while 5 kV and -15 V in negative ion mode. Capillary temperature and sheath gas flow (N_2) were, set respectively at 350ºC and 30 arbitrary units in both scan modes. Data were acquired in positive and negative MS total ion scan mode (mass scan range: *m*/*z* 100 2000) and in positive MS/MS product ion scan mode; the normalized collision energy (nce %) was optimized for each precursor ion selected: *m*/*z* 1030, 38%; 1044, 1058, 1072, 1086 and 1100, 39%; 1464, 1478, 1492 and 1506, 35%.

2.5.2. Liquid chromatography–mass spectrometry analysis

A Surveyor HPLC on line with a LCQ DECA XP Plus (Thermo Finnigan, San Jose, CA, USA) Ion Trap mass spectrometer equipped with an ESI source was employed. Separations were performed on an analytical Luna 5 µm C18, 150×4.6mm (Phenomenex, Torrance, CA) protected with a C18-Security Guard cartridge, 4 × 3.0 mm (Phenomenex). The injection volume applied was 10 µl. Mobile phase components encompassed: A -

formic acid/ ammonium formate buffer and B- acetonitrile. Moreover, lipopeptides were eluted according the following gradients: gradient 1 A: B (60: 40) for 4 min, then A: B (0: 100) over 24 min and finally 100% B over 6 min, at a flow rate of 0.8 ml min^{-1} ; gradient 2 A: B (20: 80) for 5 min, then A: B (0: 100) over 20 min and finally 100% B over 15 min, at a flow rate of 0.8 ml min^{-} LC/MS full scan positive mode was performed from *m*/*z* 100 to 2000. Alternatively, LC/ESI-MS/MS modalities were applied to the selection of precursor ions, following the conditions set during the infusion analysis.

3. Results and Discussion

3.1. Surface-active producers within *Bacillus pumilus* group species

Biosurfactant production ability was observed in most of *B. pumilus* (3 of 4 isolates) and *B. safensis* (9 of 15) isolates tested. Interestingly, none of *B. altitudinis* isolates revealed substantial surface tension reduction (Table 2) Biosurfactant producers belonging to *B. pumilus* (Bp7, Bp11 and Bp15) were recovered from contaminated health´s products of a pharmaceutical production unit. Moreover, with exception for food isolates, *B. safensis* from different origins produced biosurfactants (Bs2, Bs3, Bs13, Bs16, Bs17, Bs18, Bs19, Bs22 and Bs27). In addition, the *B. subtilis* isolate tested (Bsb28) also revealed a good reduction of surface tension.

The putative emulsifying activity (E_{24} emulsification index) of the cell-free extracts, tested using kerosene (hydrophobic compound) was also investigated (Table 2). Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble substances, the water bioavailability of such substances and also change the properties of the bacterial cell surface. Therefore, this surface activity makes surfactants excellent emulsifiers. The emulsifying activity is determined by its strength in retaining the emulsion of the hydrocarbons in water (Fracchia et al., 2012).

As expected, isolates not able to substantially decrease the superficial tension, didn't also reveal emulsifying activity. Moreover, none of the *B. pumilus* showed notable activity. Nevertheless, some of *B. safensis* isolates tested that revealed substantial surface tension reduction ability, presented emulsifying activity, e.g. animal isolates, Bs2 (E_{24} of 17.81%) and Bs3 (21.28%); cosmetic contaminants isolates, Bs18 (19.12%) and Bs19 (20.00%) and the plant growth promoter isolate Bs22 (18.67%). Nevertheless, the highest emulsifying index (the E_{24}) was observed in the cell-free extract of the non-B. pumilus group isolate tested, the *B. subtilis* isolate (40.00%). The fact that the emulsifying activity has been detected in the cell-free extract indicated that this activity was extracellular.

In order to evaluate the stability of the emulsion, cell-free extracts were also evaluated after 8 days and 3 months. Results demonstrated that only Bs3, Bs18 and Bsb28 produced stable emulsifying agents, being the remarkably E_{24} retained for Bsb28 (E_{24}=40.0). In fact, variations in the length of the fatty acids chains and differences in the amino acidic sequences (Table 4) could possibly explain the different emulsifying activity

detected. Bsb28 present a chromatogram profile correspondent to surfactin and fengycin families, while Bs3 and Bs18 with pumilacidin. To our knowledge, pumilacidin have not yet been reported so far, as presenting emulsifying activity. Nevertheless, a heteropolymer constituted by glucose, mannose, xylose, arabionose and N-acetyl glucosamine as monomers units, produced by *B. pumilus* UW-02, showed significant emulsifying activity (Chowdhury et al., 2011). Moreover, nC(14)-surfactin and anteisoC(15)-surfactin produced by a *Bacillus velezensis* H3 strain recovered from sea mud was found to evidence a good bioemulsifier funtion coupled with biosurfactant activity (Liu et al., 2010). In fact, high molecular weight biopolymers as carbohydrates, proteins, lipids and other organic and inorganic compounds, instead of low molecular weight polymers, are usually referred to as bioemulsifiers (Banat et al., 2010). In this sense, a more detailed chemically characterization should be performed in order to clarify the chemical origin of the observed emulsifier activity.

3.2. Detection of lipopetide synthethase genes by PCR

The presence of specific biosynthetic genes for the three lipopeptides families (surfactins/lichenysins, fengycins/plipastatins and iturins) commonly found in *Bacillus* spp. was evaluated by PCR amplification and sequencing.

Primary structure and genetic organization of the operons encoding the non-ribosomal peptide synthetases (NRPSs) of surfactin, lichenysin, iturin, pliplastatin and fengycin were previously described (Athukorala et al., 2009, Hsieh et al., 2004 and 2008, Nieminen et al., 2007, Tapi et al., 2010). Recently, based on the alignment of nucleic acid sequences of adenylation and thiolation domains of these NRPSs, Tapi et al. (2010) designed specific degenerated primers, targeting NRPS genes particularly involved in *Bacillus* spp. lipopeptides biosynthesis. In addition to these specific degenerated primers, we have also selected different primers sequences previously constructed against the main lipopeptide synthetases families based on different *Bacillus* spp. genomes (Athukorala et al., 2009, Hsieh et al., 2004 and 2008, Nieminen et al., 2007). Despite the majority of these lipopeptides genes being relatively conserved at the species level, which highlights their importance, they also demonstrate some dissimilarities even among closely related species (Hsieh et al., 2004), which can stresses the fact that different species can produce distinct effective biosurfactant molecules.

In the case of surfactin, one of the genes required for its biosynthesis is a large operon of 25kb, named *srfA* (Hsieh et al., 2004, Tapi et al., 2010). The *sfp* gene, encoding an enzyme belonging to the superfamily of 4´-phophopantetheinyltransferase, was mapped 4kb downstream of the *srfA* operon, and reported as the second essential gene for the surfactin production (Tapi et al., 2010).

Using the primers previously described by Hsieh et al. (2004), *sfp* gene was only detected in the *B. subtilis* isolate and the amplified fragment demonstrated 99% of homology with *sfp* gene of *Bacillus subtilis* subsp. *subtilis* 6051-HGW (GenBank accession number NC_020507.1).

Clustal analyses of different *sfp* genes available in GenBank database, including 4´-phophopantetheinyltransferase from *B. pumilus* SAFR-032 and ATCC 7061^T, and others from *Bacillus subtilis*, demonstrated a high level of divergence between them. Moreover, the primers pair used for *sfp* amplification revealed low complementarity with *B. pumilus* SAFR-032 and ATCC 7061^T *sfp* gene.

In addition, using specific degenerated primers (Tapi et al., 2010) for the surfactin family (*srf* gene), results demonstrated that except for one *B. safensis* isolate, the *srf* gene was detected in all isolates tested (Table 3), although this primers pair also amplify *lch* genes. Blast analysis of these amplicons sequences revealed 85% of homology with a non-specified non-ribosomal peptide subunit present in *B. pumilus* SAFR-032 genome (GenBank accession number NC_009848.1).

Lichenysin, a lipopetide whose structure presents the same multifunctional modular arrangement as seen in surfactin synthetase SrfA, is synthesized by a multienzyme complex, lichenysin synthetase (LchA/Lic) (32.4 kb) encoded by a lichenysin operon *lchA* (*lic*) (26.6 kb), which encoding the proteins LchAA (coding *lchAA* gene), LchAB (*lchAB*) and LchAC (*lchAC)* (Nerurkar, 2010).

No PCR amplification was obtained using primers specifically targeted to lichenysin synthetase genes *lchAA*, *lchAB* and *lchAC*, corroborating the absence of evidences of this lipopeptide in the LC/ESI-MS/MS analysis (explored below). Additionally and contrastingly with *B. subtilis*, no previous reports of production of lichenysin in *B. pumilus* group were obtained.

Fengycin is synthesized nonribosomally by five fengycin synthetases (37-kb), FenC, FenD, FenE, FenA and FenB, which are coded in the fengycin synthetase operon (Wu et

al., 2007)**.** Detection of fengycin synthetase genes using degenerated sequences of primers revealed that 55.2% (n=16) of isolates presented *fen* gene, and 15 of which also demonstrated the gene *srf*.

Similarly to *srf* gene, blast analysis of the sequenced amplicons revealed 87% of homology with a non-specified non-ribosomal peptide subunit present in *B. pumilus* SAFR-032 genome (GenBank accession number NC_009848.1). No PCR amplification was detected using primers targeted to specific fengycin synthetase genes *fenA*, *fenB* and *fenD.* Moreover, LC/ESI-MS/MS (explored below in section 3.3) revealed the presence of four homologues, C16 and C17-fengycin A and C16 and C17-fengycin B in *B. subtilis* isolate and no evidences of its production were achieved among *B. pumilus* group members.

In the case of plipastatin synthase NRPS subunits form a co-linear chain in the order ppsC-ppsD-ppsE-ppsA-ppsB (38 kb) (Batool et al., 2011). Using primers targeted to specific plipastatin synthetase gene *pps*, no PCR product was detected in the isolates tested.

The iturin A operon (38 kb) is composed of four open reading frames: *itu*D, *itu*A, *itu*B, and *ituC*. The *ituD* gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production. The *lpa*-14 gene encodes the 40-phosphopantheteinyl transferase required for the maturation of the template enzyme of iturin A. Thus, both the *ituD* gene and the *lpa*- 14 gene play leading roles in the production of iturin A (Hsieh et al., 2008).

No PCR amplification was detected with a set of two oligonucleotide primer pairs specific to the regions within *ituD* and *lpa*-14 genes of iturin A, respectively. Despite Hsieh et al. (2008) revealed that these set of primers could be applied to find different species or genera that contain homologous genes for *ituD* and/or *lpa*-14, they also not provided any amplification in *B. pumilus* ATCC 7061 (Hsieh et al., 2008).

Indeed, the degenerated primer sets used to detect the presence of surfactin and fengycin families suggests a genetic potential of *B. pumilus* group isolates to produce lipopeptides. Nevertheless, surfactin and fengycin synthetases genes were detected in some isolates showing surface tension properties incompatible with the production of a biosurfactant (Table 3), e. g. Bp ATCC 14884, Bs5, 23 Bs, Bs24, Bs 25, Bs 31, Bs 33 and all *B. altitudins* isolates.

In fact, the detection of a particular lipopeptide biosynthetic gene in a bacterial strain could not signify the function of its operon and consequently, its production (Mootz et al., 2001). Although we can't discard the possibility of a gene mutation preventing lipopeptide production northat the appropriated culture conditions for expression of the correspondent compound were not applied.

3.3. Lipopeptides characterization and structure elucidation by LC/ESI-MS/MS

From the 13 biosurfactant-producing isolates, 6 were selected to biochemical and structure characterization based on the species producing, surface tension properties, E_{24} activity and the presence of lipopetides biosynthetic genes detected by PCR. In the mass spectra of *B. pumilus*, *B. safensis* and *B. subtilis* studied, different types of lipopeptides, or its variants prevail, as summarized in Table 4.

For the *B. safensis* Bs18 extract, full-scan mass spectra (Fig. 1) through ESI-MS direct infusion analysis, operating in positive full scan mode, showed one cluster of peaks with 14 Da difference on its molecular ion species.

Four main signals, corresponding to the sodiated molecules in the positive ion mode $[M+Na]^+$ at *m/z* 1058, 1072, 1086 and 1100 were detected. Moreover, analyses in negative full scan mode revealed the presence of four main peaks at *m/z* 1034, 1048, 1062 and 1076 corresponding to deprotonated molecules $[M-M]^-$. Indeed, molecular weights for the four peaks can be attributed, specifically 1035, 1049, 1063 and 1077 Da, which are similar with the molecular weights reported for surfactin and pumilacidin lipopeptides. Then, LC-ESI-MS/MS analysis was performed according gradient 2, on precursor ions $[M+Na]^+$ at *m/z* 1058, 1072, 1086 and 1100.

LC-ESI-MS/MS from precursor ions $[M+Na]^+$ at *m/z* 1072 and 1100 showed one main peak for each precursor ion, at retention times (Rt) of 18.03 and 23.01 min, respectively. Product ion spectra (Fig. 2a) from *m/z* 1072 resulted in the following series of ions: *m/z* 1054, 959, 828, 731, 721, 608 and 477. Peak ion detected at *m/z* 1054 correspond to the loss of water (-18 Da) from the *m/z* 1072. Moreover, the remaining product ions were derived from the initial opening of the lactone ring,

Thus, the first series included the fatty acid chain and the N-terminal product ions at *m/z* 959, 828 and 731, corresponding to the losses of Leu (-113 Da), Leu-Leu-H_2O (-244 Da)

and Leu-Leu-Asp (-341 Da) from the *m/z* 1072. In addition, the second one enclosed the peptidic moiety inside the C-terminal product and corresponds to the ions peaks at m/z 721, 608 and 477. Ion at *m/z* 721 correspond to the loss of C_{15}–β-hydroxyl fatty acid chain -Glu (-351 Da) from the precursor ion, following double hydrogen transfer (DHT) mechanism of the ester bond of the cyclic skeleton and cleavage of one **C**-terminal amino acid residue (Yang et al., 2006). Moreover, ion at *m/z* 608 match to the successively loss of Leu (-113 Da) and ion at *m/z* 477 to the loss of Leu-Leu-H_2O (-244 Da).

LC-ESI-MS/MS spectra analysis from precursor ion $[M+Na]^+$ at 1100 (Rt 23.01 min) (Fig. 2b) revealed, as above mentioned, two product ion series, comprising *m/z* 1082, 987, 856 and 759 related to the fatty acid chain, and ions at *m/z* 721, 608 and 477. Within the first series product ions belonging to *m/z* 1100 differ of 28 Da from those belonging to *m/z* 1072, confirming the difference of a -CH_2–CH_2 in the side chain. Furthermore, in the second series all product ions are identical to the previous mentioned, thus, corresponding to the same amino acid sequences.

Indeed, these ion signals are in accordance with two pumilacidin-Leul7 homologues, respectively C15-Glu/Leu/Leu/Leul/Asp/Leu/Leu and C17- Glu /Leu/Leu/Leul/Asp/Leu/Leu, (Fig. 3a), which can be related with pumilacidin A ($[M+Na]^+$ 1073 *m/z*) or F ($[M+Na]^+$ 1073 *m/z*) or G ($[M+Na]^+$ 1073 *m/z*) and with C ($[M+Na]^+$ 1101 *m/z*) types, respectively (Burch et al., 2011, Naruse et al., 1990).

Interestingly, the LC-ESI-MS/MS analysis from the precursor ions $[M+Na]^+$ at *m/z* 1058 and 1086 showed three isobaric peaks for each precursor ion, at Rt 15.98, 16.56 and 17.85 and also 20.46, 21.25 and 22.84 min, respectively. From the precursor ion $[M+Na]^+$ at *m/z* 1058 the product ion spectra of the peak at Rt 15.98 min also demonstrates two product ion series. In the first series (*m/z* 1040, 945, 814) related to the fatty acid chain, product ion belonging to *m/z* 1058 differed of 14 Da from those belonging to m/z 1072, also confirming the difference of a –CH_2 in the side chain. Within the second series (*m/z* 721, 608, 477) all product ions are equal and consequently the amino acid sequences the same. Additionally, this signal was in accordance with C14-Glu/Leu/Leu/Leul/Asp/Leu/Leu, the third homologue of pumilacidin-Leu7 molecules.

Analysis of ion spectra of the peak at Rt 16.56 min (Fig. 4a) reveal ions products at *m/z* 1040, 959, 828, 731, 707, 594 and 463. Ion at *m/z* 1040 correspond to the loss of water (-18 Da) from *m/z* 1058 and the remaining product ions were deriving, as before, from the initial opening of the lactone ring. In addition, the first series contain the fatty acid chain

and the N-terminal product ions at *m/z* 959, 828 and 731, corresponding to the losses of Val (-99 Da), Val-Leu-H_2O (-230 Da) and Val-Leu-Asp (-327 Da) from *m/z* 1058, respectively. The second series encompass a different peptidic moiety inside the C-terminal product ions at *m/z* 707, 594 and 463, where ion at *m/z* 707 correspond to the loss of C15 –β-hydroxyl fatty acid chain-Glu (-351 Da) from the precursor ion, ion at *m/z* 594 to the successively loss of Leu (-113 Da) and m/z 463 to the loss of Leu-Leu-H_2O (-244 Da). In fact, these signals are in agreement with the C15-Glu/Leu/Leu/Leul/Asp/Leu/Val, pumilacidin-Val7 variant (Fig. 3 b).

Related to resulting peak at Rt 17.85 min (Fig. 5a) ions product at *m/z* 1040, 945, 814, 618, 707, 594 and 463 were detected. Ion at *m/z* 1040 correspond to the loss of water (-18 Da) from *m/z* 1058. The resulting series contained the fatty acid chain and the N-terminal product ions at *m/z* of 945, 814 and 618 corresponding to the losses of Leu (-113 Da), Leu-Leu-H_2O (-244 Da) and Leu-Leu-Asp-Val (-440) from *m/z* of 1058, respectively, in the first series, and in the second, enclose different peptidic moieties inside the C-terminal products at *m/z* 707, 594, and 463. Moreover, ion at *m/z* 707 corresponds to the loss of C15–β-hydroxyl fatty acid chain-Glu (-351 Da) from the precursor ion, *m/z* 594 to the successively loss of Leu (-113 Da) and m/z of 463 to the loss of Leu-Leu-H_2O (-244 Da). These signals are in accordance with the C15-Glu/Leu/Leu/Vall/Asp/Leu/Leu homologue belonging to surfactin molecules (Pecci et al., 2010) (Fig. 3c).

Precursor ion analysis at *m/z* 1086 showed three peaks at Rt 20.46, 21.25 and 22.84 min. Rt 20.46 min product ion spectra resulting, as before, into two product ion series. The peptidic series (*m/z* 721, 608 and 477) is relative to pumilacidin and the aliphatic series (*m/z* 1068, 973, 842, 745) differ of –CH2 of the previous homologue. These signals are in conformity with the C16-Glu/Leu/Leu/Leul/Asp/Leu/Leu, the fourth homologue of pumilacidin-Leu7.

Rt 21.25 min product ion spectra, also show two product ion series (Fig. 4b), comprising the peptidic one, at *m/z* 707, 594 and 463, which is relative to the variant of Val7 of pumilacidin molecule and the aliphatic one, enclosing *m/z* 1068, 987, 856 and 759 which differ of -CH2–CH2 of the previous homologue. C17-Glu/Leu/Leu/Leu/Asp/Leu/Val, the second homologue of pumilacidin-Val7 match these masse signals observed.

Finally, at Rt 22.84 min the product ion spectra (Fig. 5b) revealed the presence of the peptidic series (*m/z* 707, 594 and 463) relative to surfactin molecule, and the aliphatic series (*m/z* 1068, 973, 842 and 745) differing in -CH2–CH2 of the previous homologue,

which are in accordance with the C17-Glu/Leu/Leu/Vall/Asp/Leu/Leu, the second homologue of surfactin molecule.

In fact, detection of chemical differences in pumilacidin and surfactin were previously observed to occur in amino acid position 4 or 7 of the peptidic ring. Thus, pumilacidin variants are consistent with Leu4, Leu7 or Val7 surfactins (Morikawa et al., 1992).

The relative abundance of the three surfactin-like biosurfactants variants presented in *B. safensis* isolates analyzed (Bs2, Bs22, Bs18 and BS27) were inferred from sum of the product ions attributed to the relative precursor ions $[M+Na]^+$. Beyond *B. safensis* Bs18, all the remaining isolates also presented the same relative abundance on its surfactin-like variants produced, being detected an average of 90% in pumilacidin variants and of 10% in surfactin ones (Table 4).

These observations prompt us to suggest that the production of pumilacidin-Leu7, or its variant Val7, together with surfactin seems to be *B. safensis* species-specific and thus, can be related to its adaptability and defense mechanisms into different ecological niches (Branquinho et al., 2014).

Related to *B. pumilus* species analyzed, full-scan mass spectra derived from Bp11, demonstrated that pumilacidin variant Leu7 and variant Val7 were present (Table 4) with a relative abundance average of 94 and 3%, respectively for each one. Characterization of *B. pumilus* Bp11 extracts through ESI-MS direct infusion analysis operating in positive full scan mode, showed one cluster of peaks with 14 Da difference on its molecular ion species. Four main signals at 1058, 1072, 1086 and 1100 *m/z* were detected, which correspond to the sodiated molecules $[M+Na]^+$. The positive LC-ESI-MS/MS analysis on the sodiated molecule at *m/z* of 1058, 1072, 1086 and 1100 performed on *B. pumilus* extract, and similar to previously observed to *B. safensis* isolates, revealed that these signals were in according with the four homologues of pumilacidin-Leu7 variants compounds, respectively C-14, C-15, C-16 and C-17 and also C-15 and C-17 pumilacidin-Val7 variant.

In contrast with *B. safensis*, *B. pumilus* isolates seems to be able to produce just one type of surfactin-like compounds, specifically pumilacidin. Structure-activity relationships should be further conducted, in order to explain in what dimension these differences can interfere with the activity of these compounds.

Characterization of *B. subtilis* Bs28 extracts through ESI-MS direct infusion analysis operating in positive full scan mode, showed two clusters of peaks with 14 or 28 Da of difference in their molecular ion species (Fig. 6), which in accordance with other work performed by our research team (Pecci et al., 2010), correspond with spectral analysis of surfactin and fengycin compounds. Surfactin is represented by three main mass signals at $[M+Na]^+$, namely at *m/z* of 1030, 1044 and 1058. Results derived from our LC/ESI-MS/MS analysis performed with gradient 1 are in accordance with the presence of three surfactin homologues, e.g. C13- Glu/Leu/Leu/Val/Asp/Leu/Leu, C14-Glu/Leu/Leu/Val/Asp/Leu/Leu and C15-Glu/Leu/Leu/Val/Asp/Leu/Leu and none of the pumilacidin variants detected for *B. safensis* or *B. pumilus* were here achieved. Moreover, fengycin was characterized by four mass signals at $[M+H]^+$, namely at *m/z* of 1478 and 1464 for fengycin A and *m/z* of 1492 and 1506 for fengicyn B (Pecci et al., 2010). Our spectral results are in agreement with the previously description, revealing the presence of C16 and C17 homologues of fengycin A and C16 and C17 homologues of fengycin B. The relative abundance achieved in *B. subtilis* Bsp28 was of 90% for surfactin and of 10% for fengycin.

Since biosurfactants production depends of the type and amounts of carbon source and of other nutrients present in the screening culture medium (Soberón-Chávez, 2011), it will consequently influence the composition content of the mixture and the amount of compounds produced. In fact, it is possible that other type of biosurfactants could be produced by these *Bacillus* spp., nevertheless the cultural optimization and screening conditions were not the aim of the present study. Therefore, a more comprehensive screening can be further performed in order to obtain new potential biosurfactants with putative possible different applications. LB broth has been reported as the culture medium more extensively used for *Bacillus* biosurfactant production (Rivardo et al., 2009), and therefore it was accepted and applied for our screening evaluation.

The distribution of distinct combinations of these molecules within *Bacillus pumilus* group species recovered from different origins and geographic locations, suggested that biosurfactant production may represent an important survival tool of each species in a specific environment. Moreover, the differences observed in the length of the fatty acid chains and in the amino acid composition of the biosurfactants and its species-specificity, can suggest considerable consequences on its activity and consequently on its possible applications.

4. Conclusion

The specificity of biosurfactants molecules produced by species belonging to *B. pumilus* group supports the speciation recently recognized within this taxonomic group (Branquinho et al., 2014, Liu et al., 2013) and might conferred a distinct niche adaptability. Contrasting with *B. subtilis*, pumilacidin seems to be the more predominant variant in *B. pumilus* group species. Further studies exploring the properties of the different biosurfactants characterized could uncover interesting effects broadening potential applications of these compounds. Moreover, our data highlights the failure in the prediction of biosurfactant production based merely on the detection of lipopeptides synthetase genes by a PCR approach.

Table 1. Primers sequences and amplification conditions for screening the main families of lipopeptides biosynthetic genes in *Bacillus* spp.

Lipopetides	Gene	Primer name	Primer sequences (5´to 3´)	Annealing temperature (ºC)	PCR product size expected	References
Surfactin/Lichenysin family						
Surfactin	*srf/lch*[a]	As1-F	CGCGGMTACCGVATYGAGC	43	419/422/425/431	Tapi et al., 2010
		Ts2-R	ATBCCTTTBTWDGAATGTCCGCC			
	sfp	sfp-f	ATGAAGATTTACGGAATTTA	46	675	Hsieh et al., 2004
		sfp-r	TTATAAAAGCTCTTCGTACG			
Lichenysin	*lchA*A	LicA-f	GTGCCTGATGTAACGAATG	60	735	Nieminen et al., 2007
		LicA-r	CACTTCCTGCCATATACC			
	*lchA*B	LicB2-f	TGATCAGCCGGCCGTTGTCT	60	904	Nieminen et al., 2007
		LicB2-r	GGCGAATTGTCCGATCATGTCC			
	*lchA*C	LicC-f	GCCTATCTGCCGATTGAC	57	1195	Nieminen et al., 2007
		LicC-r	TATATGCATCCGGCACCA			
Fengycin/Plipastation family						
Fengycin	*fen*[a]	Af2-F	GAATAYMTCGGMCGTMTKGA	45	443/452	Tapi et al., 2010
		Tf1-R	GCTTTWADKGAATSBCCGCC			
	*fen*A	FENA1F	GACAGTGCTGCCTGATGAAA	62	964	Athukorala et al., 2009
		FENA1R	GTCGGTGCATGAAATGTACG			
	*fen*B	FENB2F	CAAGATATGCTGGACGCTGA	62	964	Athukorala et al., 2009
		FENB2R	ACACGACATTGCGATTGGTA			
	*fen*D	FEND1F	TTTGGCAGCAGGAGAAGTTT	62	964	Athukorala et al., 2009
		FEND1R	GCTGTCCGTTCTGCTTTTTC			
Pliplastatin	*pps*[a]	Ap1-F	AGMCAGCKSGCMASATCMCC	58	893/959/929	Tapi et al., 2010
		Atp1-R	GCKATWWTGAARRCCGGCGG			
Iturin family						
Iturin	*lpa*-14	lpa-14f	ATGAAAATTTACGGAGTATA	50	675	Hsieh et al., 2008
		lpa-14r	TTATAACAGCTCTTCATACG			
	*itu*D	ituD-f	ATGAACAATCTTGCCTTT TTA	50	1203	Hsieh et al., 2008
		ituD-r	TTATTTTAAAATCCGCAATT			

[a] degenerated primers

Table2. Surface tension and emulsification properties of *Bacillus* spp. studied.

***Bacillus* species**	**Bacterial isolates**	**Origin/Product**	**Year/Location**	**Surface tension**						**E_{24} (%)**		
				Qualitative (mm)		Quantitative (mN/m)						
				Cell-free supernatant		Cell-free supernatant		Purified Biosurfactant (500ug/mL)		Cell-free supernatant		
				Average	SD	Average	SD	Average	SD	24 h	8 days	3 months
B. pumilus	Bp ATCC14884			3.00	0.00	58.29	0.40			3.90	2.60	2.60
	Bp7	Health´s product contaminants (n=3)	2005/Portugal[1]	17.67	1.44	38.69	0.21			4.29	4.29	2.86
	Bp11*		2005/Portugal[1]	18.67	1.04	35.24	0.39	31.4	0.27	1.30	1.30	1.30
	Bp15		2005/Portugal[1]	17.83	0.76	36.38	0.17			6.67	5.33	5.33
B. altitudinis	Ba6	Health´s product ´ contaminants (n=5)	2005/Portugal[1]	4.50	0.50	45.92	0.50			2.67	2.67	1.33
	Ba8		2005/Portugal[1]	6.00	0.50	41.64	0.37			1.33	1.33	1.33
	Ba9		2005/Portugal[1]	6.17	0.29	45.41	0.15			1.35	1.35	0.00
	Ba12		2005/Portugal[1]	5.00	0.50	47.19	0.29			2.86	2.86	1.43
	Ba14		2005/Portugal[1]	8.83	0.29	43.66	0.55			0.00	0.00	0.00
	Ba26	Food´s contaminants/rice (n=1)	2006/Norway[2]	6.67	0.29	51.24	0.41			0.00	0.00	0.00
	Ba20	Plant Growth Promoters (PGPR) (n=2)	1996/USA[4]	6.00	0.50	47.66	0.30			1.30	1.30	0.00
	Ba21		1997/USA[4]	5.83	0.29	46.40	0.17			2.67	2.67	0.00

	Ba30	Food/beans (n=1)	2003/Africa[5]	7.17	0.58	43.64	0.36			1.43	1.43	0.00
Bacillus safensis	Bs2*	Animals Gastropods (n=2)	2005/Portugal[1]	18.83	0.29	36.18	0.31	29.5	0.13	17.81	17.81	8.22
	Bs3		2007/Portugal[1]	19.33	0.76	35.37	0.25			21.28	21.28	21.28
	Bs13	Health´s product contaminants (n=3)	2005/Portugal[1]	18.10	0.50	33.53	0.34			2.70	2.70	2.70
	Bs16		2005/Portugal[1]	18.33	2.36	34.19	0.26			3.85	3.85	2.56
	Bs17		2005/Portugal[1]	18.67	2.75	35.36	0.12			7.14	5.71	2.86
	Bs5	Cosmetic´s contaminants (n=4)	2002/Portugal[1]	4.00	0.50	49.67	0.32			2.86	2.86	1.43
	Bs18*		2002/Portugal[1]	19.17	0.29	34.32	0.20	33.4	0.29	19.12	19.12	16.18
	Bs19		2002/Portugal[1]	17.00	1.80	33.60	0.22			20.00	20.00	13.33
	Bs27*		2002/Portugal[1]	24.17	2.47	30.50	0.18	31.5	0.33	10.39	10.39	2.60
	Bs24	Food´s contaminants/salame (n=3)	2004/Italy[3]	3.50	0.50	46.51	0.42			0.00	0.00	0.00
	Bs25		2004/Italy[3]	4.00	0.50	44.40	0.34			6.49	6.49	2.60
	Bs33		2004/Italy[3]	6.00	0.50	42.59	0.28			1.33	1.33	1.33
	Bs22*	Plant Growth Promoters (PGPR) (n=2)	1997/USA[4]	20.10	0.50	32.27	0.13			18.67	16.00	6.67
	Bs23		1997/USA[4]	6.17	0.29	44.30	0.21			12.86	12.00	6.67
	Bs31	Food/beans (n=1)	2003/Africa[5]	7.00	0.50	40.28	0.33			11.54	11.54	2.56
Bacillus subtilis	Bsb28*	Health´s product contaminants (n=1)	2005/Portugal[1]	19.67	1.15	30.53	0.31	30.4	0.27	40.00	40.00	40.00

* isolates characterized by LC/ESI-MS/MS

SD – standard deviation

Table 3. PCR amplification results for main families of lipopeptides in *B. pumilus*, *B. safensis*, *B. altitudinis* and *B. subtilis* isolates.

Species	Isolates	Surfactin/Lichenysin family					Fengycin/Plipastatin family					Iturin family	
		srf/lch	*sfp*	*lchAA*	*lchAB*	*lchAC*	*fen*	*fenA*	*fenB*	*fenD*	*pps*	*lpa*-14	*ituD*
B. pumilus	Bp ATCC14884	+	-	-	-	-	-	-	-	-	-	-	-
	Bp7	+	-	-	-	-	+	-	-	-	-	-	-
	Bp11	+	-	-	-	-	+	-	-	-	-	-	-
	Bp15	+	-	-	-	-	+	-	-	-	-	-	-
B. altitudinis	Ba6	+	-	-	-	-	-	-	-	-	-	-	-
	Ba8	+	-	-	-	-	+	-	-	-	-	-	-
	Ba9	+	-	-	-	-	-	-	-	-	-	-	-
	Ba12	+	-	-	-	-	-	-	-	-	-	-	-
	Ba14	+	-	-	-	-	+	-	-	-	-	-	-
	Ba26	+	-	-	-	-	-	-	-	-	-	-	-
	Ba30	+	-	-	-	-	+	-	-	-	-	-	-
	Ba20	+	-	-	-	-	-	-	-	-	-	-	-
B. safensis	Bs2	+	-	-	-	-	-	-	-	-	-	-	-
	Bs3	+	-	-	-	-	-	-	-	-	-	-	-
	Bs5	-	-	-	-	-	+	-	-	-	-	-	-
	Bs13	+	-	-	-	-	-	-	-	-	-	-	-
	Bs16	+	-	-	-	-	+	-	-	-	-	-	-

	Bs17	+	-	-	-	-	-	-	-	-	-	-	-
	Bs18	+	-	-	-	-	+	-	-	-	-	-	-
	Bs19	+	-	-	-	-	+	-	-	-	-	-	-
	Bs21	+	-	-	-	-	-	-	-	-	-	-	-
	Bs22	+	-	-	-	-	+	-	-	-	-	-	-
B. safensis	Bs23	+	-	-	-	-	+	-	-	-	-	-	-
	Bs24	+	-	-	-	-	+	-	-	-	-	-	-
	Bs25	+	-	-	-	-	-	-	-	-	-	-	-
	Bs27	+	-	-	-	-	-	-	-	-	-	-	-
	Bs31	+	-	-	-	-	+	-	-	-	-	-	-
	Bs33	+	-	-	-	-	+	-	-	-	-	-	-
B. subtilis	Bsp28	+	+	-	-	-	+	-	-	-	-	-	-

Table 4. LC/ESI-MS/MS analysis of pumilacidin, surfactin and fengycin homologues produced by *B. safensis, B. pumilus* and *B. subtilis* isolates.

Species Isolates		Pumilacidin Leu7 homologues R-Pumilacidin Leu7				Pumilacidin Val7 homologues R-Pumilacidin Val7				Surfactin homologues R-surfactin				Fengycin A/B homologues R-fengycin A / R-fengycin B			
		MW	R	$M+Na]^+$ m/z	Relative %	MW	R	$M+Na]^+$ m/z	Relative %	MW	R	$M+Na]^+$ m/z	Relative %	MW	R	$M+H]^+$ m/z	Relative %
B. safensis	Bs2	1035	C14-	1058	84	1035	C15-	1058	6	1035	C15-	1058	10				
		1049	C15-	1072													
		1063	C16-	1086		1063	C17-	1086		1063	C17-	1086					
		1077	C17-	1100													
	Bs27	1035	C14-	1058	86	1035	C15-	1058	6	1035	C15-	1058	8				
		1049	C15-	1072													
		1063	C16-	1086		1063	C17-	1086		1063	C17-	1086					
		1077	C17-	1100													
	Bs18	1035	C14-	1058	83	1035	C15-	1058	5	1035	C15-	1058	12				
		1049	C15-	1072													
		1063	C16-	1086		1063	C17-	1086		1063	C17-	1086					
		1077	C17-	1100													
	Bs22	1035	C14-	1058	87	1035	C15-	1058	6	1035	C15-	1058	7				
		1049	C15-	1072													
		1063	C16-	1086		1063	C17-	1086		1063	C17-	1086					
		1077	C17-	1100													
B. pumilus	Bp11	1035	C14-	1058	94	1035	C15-	1058	3								
		1049	C15-	1072													
		1063	C16-	1086		1063	C17-	1086									
		1077	C17-	1100													
B. subtilis	Bsb28									1007	C13-	1030	90	1463	C16 Fengicyn-A	1464	10
														1477	C17 Fengicyn-A	1478	
										1021	C14-	1044		1491	C16 Fengicyn-B	1492	
										1035	C15-	1058		1505	C17 Fengicyn-B	1506	

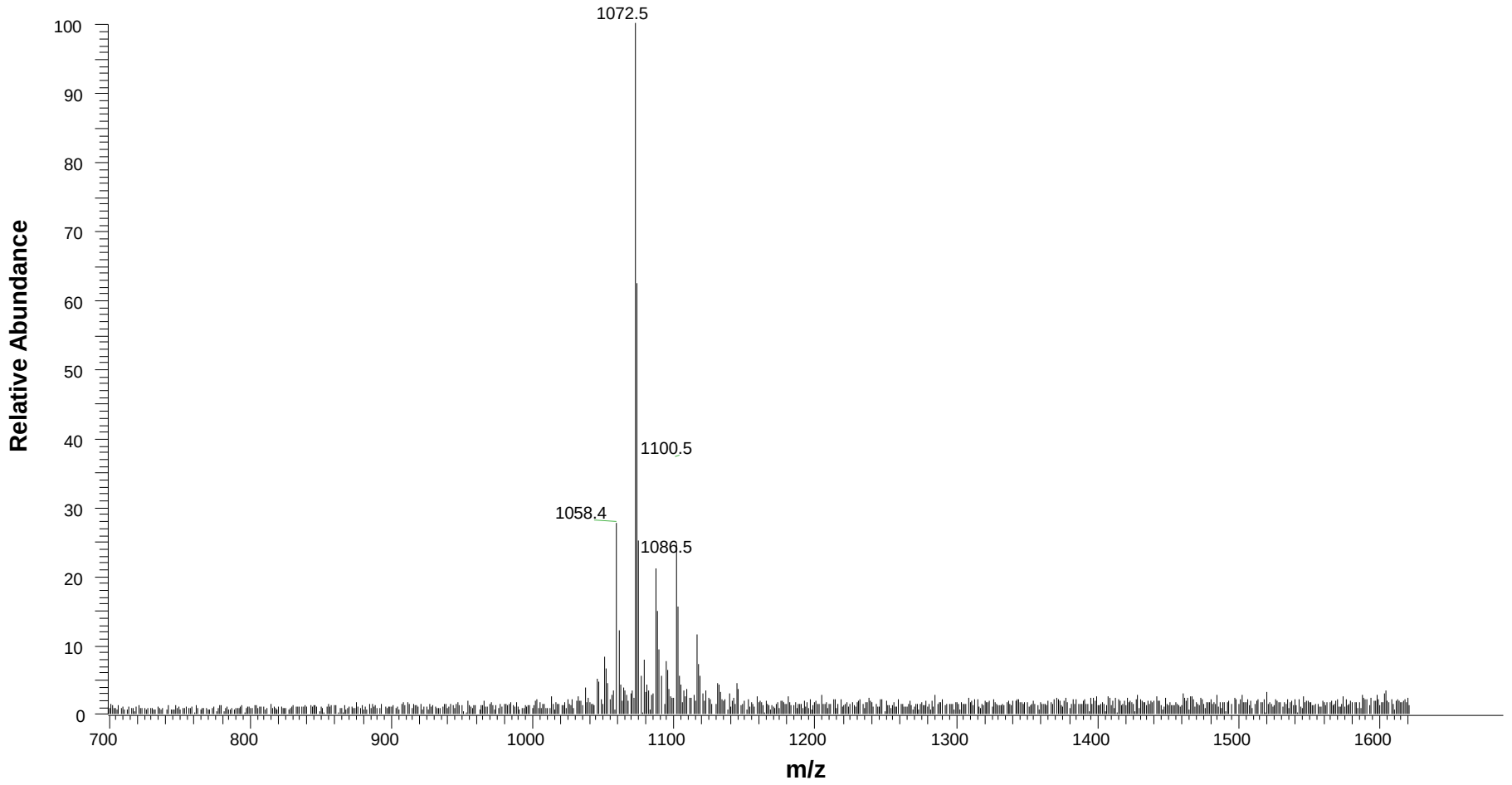


Figure 1. Full-scan mass spectra of Bs18 extract through (+) ESI-MS direct infusion analysis. Cluster of pumilacidin are shown in *m/z* 1000-1150.

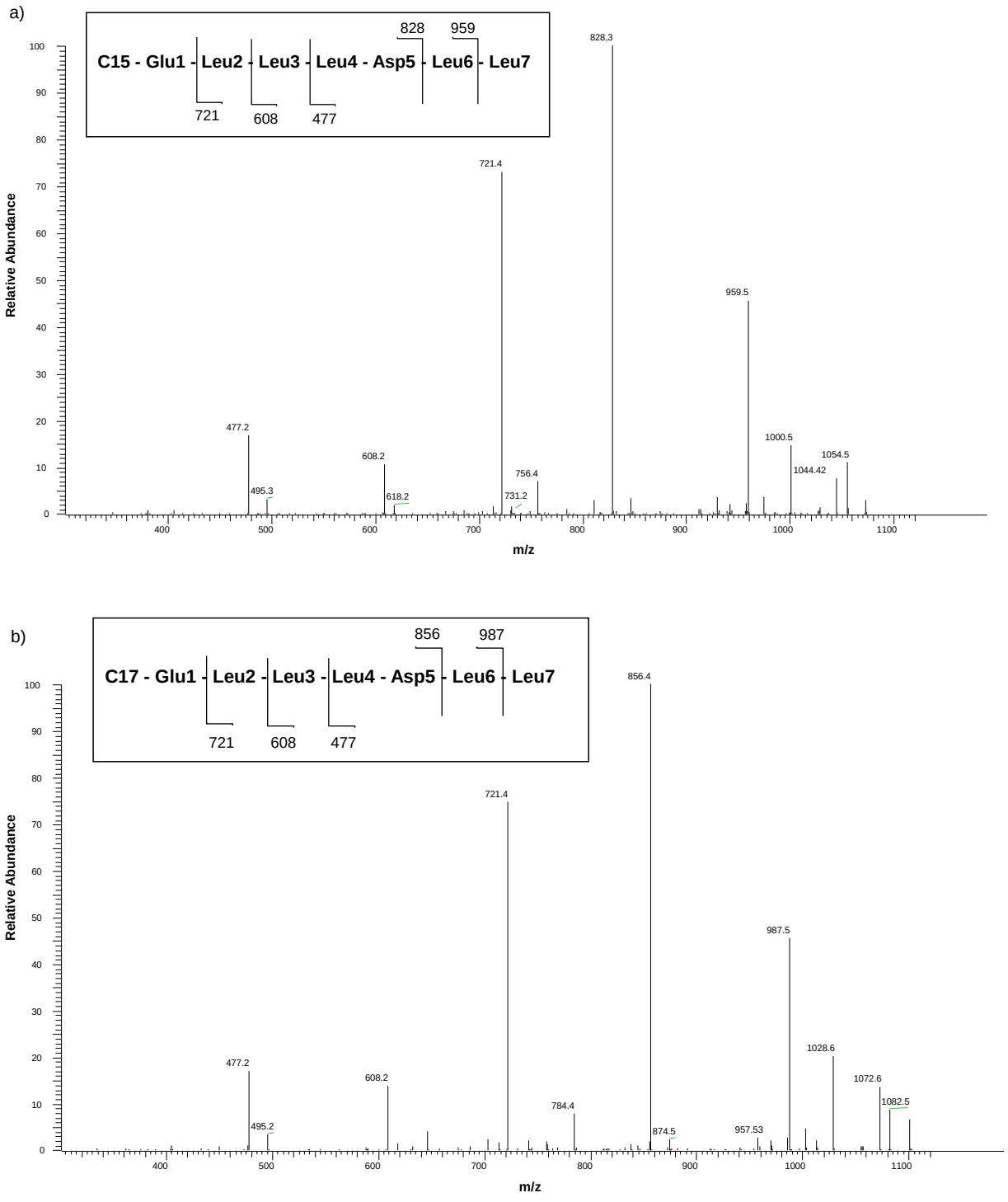


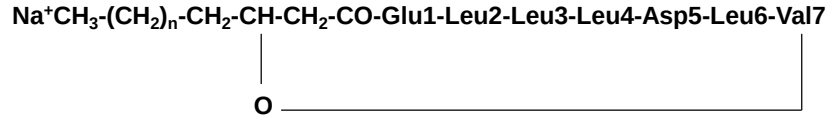
Figure 2. Product ion spectra of the sodiated molecules $[M + Na]^+$ of Bs18 pumilacidin group at *m/z* (a) 1072 (at Rt 18.03 min) and (b) 1100 (at Rt 23.01 min). Black boxes displayed cyclic structures and proposed fragmentations of (a) C15 pumilacidin Leu7 variant and (b) C17 pumilacidin Leu7 variant.

a) Pumilacidin Leu7

n=10 C15-**Glu1-Leu2-Leu3-Leu4-Asp5-Leu6-Leu7**

n= 12 C17-**Glu1-Leu2-Leu3-Leu4-Asp5-Leu6-Leu7**

b) Pumilacidin Val7



n=10 C15-**Glu1-Leu2-Leu3-Leu4-Asp5-Leu6-Val7**

n= 12 C17-**Glu1-Leu2-Leu3-Leu4-Asp5-Leu6-Val7**

c) Surfactin

n= 10 C15-**Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7**

n= 12 C17-**Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7**

Figure 3. Total cyclic structures and respective homologues of (a) Pumilacidin Leu7 variant, (b) Pumilacidin Val7 variant and (c) Surfactin.

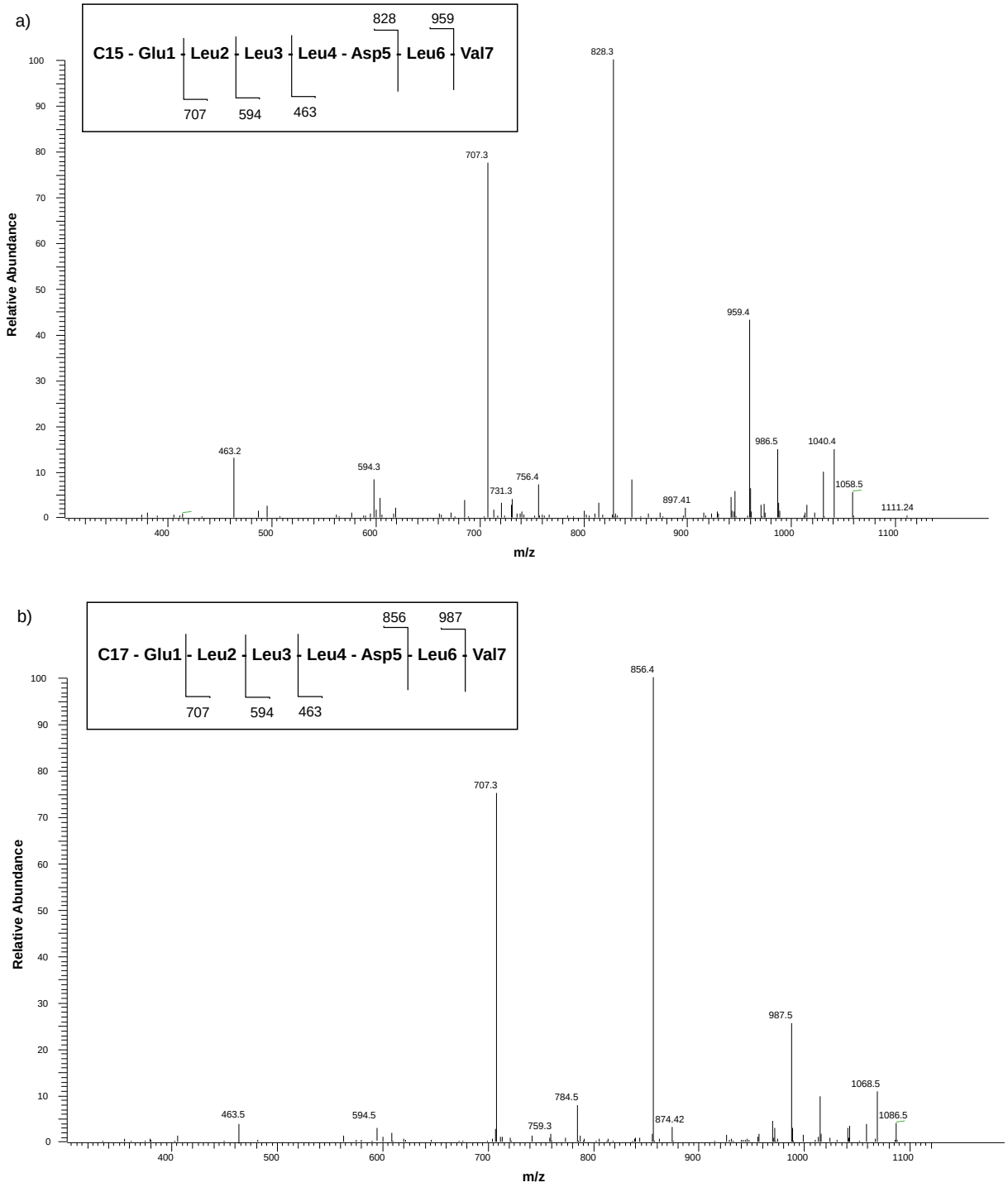


Figure 4. Product ion spectra of the sodiated molecules $[M + Na]^+$ of Bs18 pumilacidin group at *m/z* (a) 1058 (at Rt 16.56 min) and (b) 1086 (at Rt 21.25 min). Black boxes displayed cyclic structures and proposed fragmentations of (a) C15 pumilacidin Val7 variant and (b) C17 pumilacidin Val7 variant.

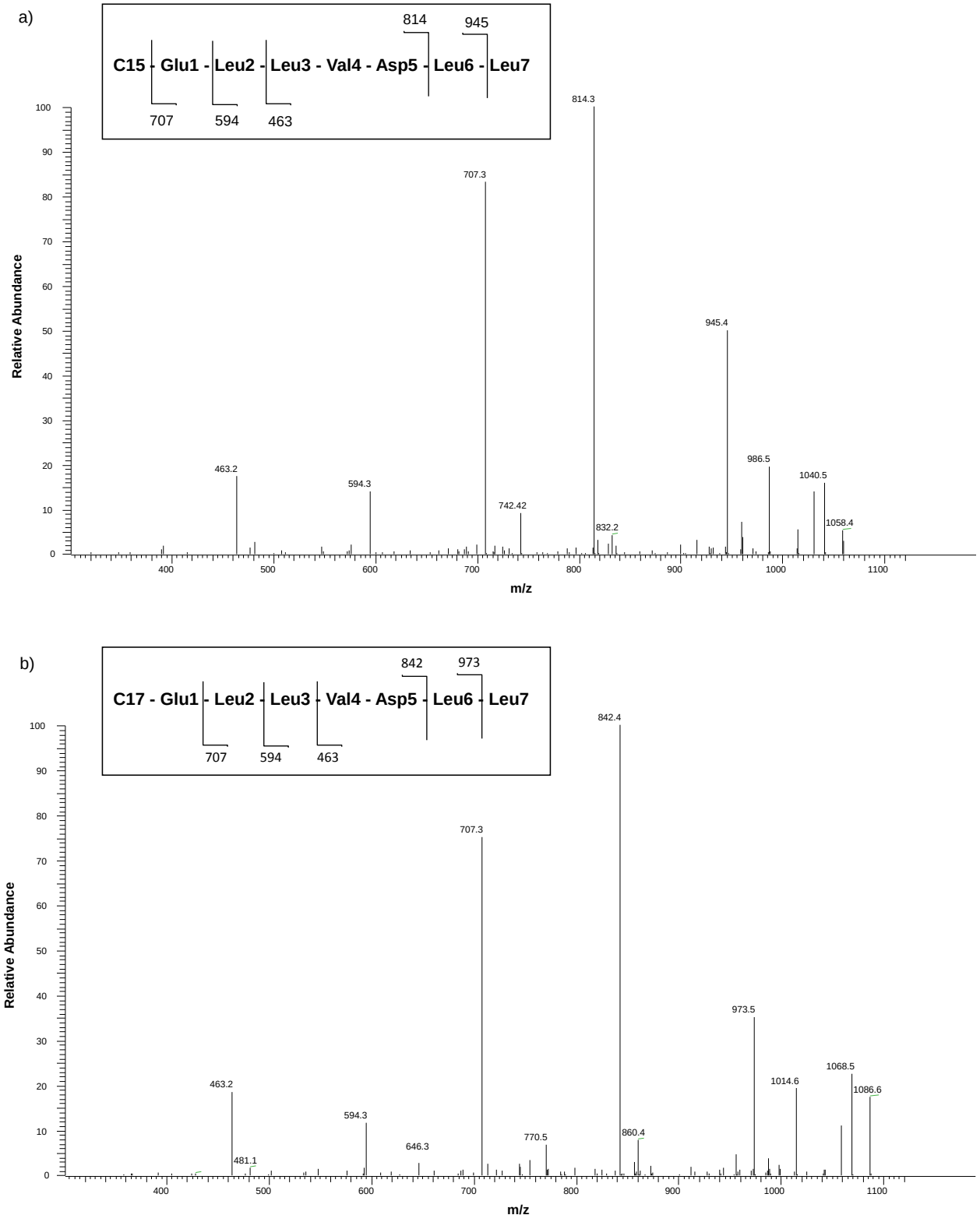


Figure 5. Product ion spectra of the sodiated molecules $[M + Na]^+$ of Bs18 pumilacidin group at *m/z* (a) 1058 (at Rt 17.85 min) and (b) 1086 (at Rt 22.84 min). Black boxes displayed cyclic structures and proposed fragmentations of (a) C15 pumilacidin Val4 variant and (b) C17 pumilacidin Val4 variant.

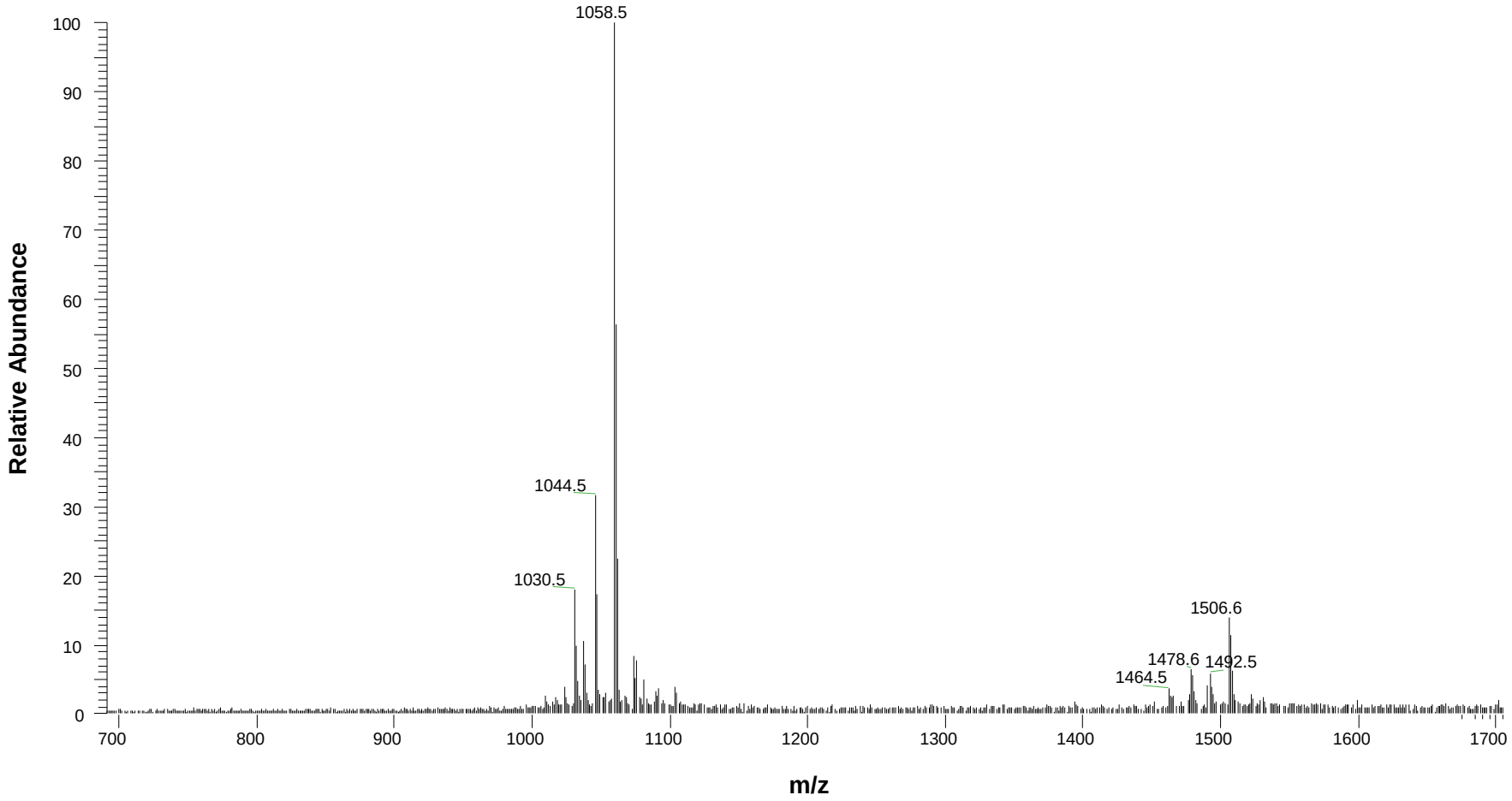


Figure 6. Full-scan mass spectra of Bsp28 extract through (+) ESI-MS direct infusion analysis. Clusters of surfactin and fengycin are shown in the ranges of m/z 1000–1100 and 1450–1550, respectively.

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4.2. Linezolid resistant ST2/CC5 *Staphylococcus epidermidis* – biofilm producer

Publication:

Emergence of Methicillin and Linezolid Resistant ST2/CC5 *Staphylococcus epidermidis,* Portugal, 2012

This study, reports the emergency of clinical methicillin and linezolid *Staphylococcus epidermidis* strains in Portugal, which belong to the worldwide successful clonal lineage ST2/CC5 (former CC2), also including the ability of *S. epidermidis* from patient 1 adhere to abiotic surfaces as a biofilm form. This isolate was used in the study enclosed in section 3.3. The data described in this manuscript also highlight the need for strict infection control procedures along with revision of therapeutic strategies to preserve linezolid therapeutic effectiveness.

Emergence of Methicillin and Linezolid Resistant ST2/CC5 *Staphylococcus epidermidis,* Portugal, 2012

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Running title: Linezolid resistant ST2/CC5 *S. epidermidis.*

Keywords: *Staphylococcus epidermidis*, linezolid resistance, biofilm, outbreak, 23S rRNA mutations, L3 protein mutations

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Emerging Infectious Diseases

Linezolid is considered a therapeutic option for skin/soft tissue infections and pneumonia due to multidrug resistant (MDR) gram-positive bacteria (e.g., *Staphylococcus* spp.), which in Portugal occurs in significant rates comparing with other European countries (http://www.ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf). *Staphylococcus epidermidis* are human skin and mucosal commensal bacteria causing different human infections, mostly linked to indwelling medical devices. Their ability to acquire antibiotic resistance and produce biofilm can seriously compromise the success of human therapy, with methicillin resistance rates exceeding 70% in many institutions worldwide (*1*). Linezolid resistance (LinR) among *S. epidermidis* has been described at low rates in different Continents, associated with mutations in the central loop of 23S rRNA V domain or ribosomal proteins (L3, L4 and L22) and with the acquisition of *cfr* gene codifying for a ribosomal methyltransferase (*1,2,3*). In Portugal, only one LinR *S. epidermidis* recovered from a dog presenting severe bilateral otitis was described (*4*). This study reports the emergence of nosocomial methicillin and LinR *S. epidermidis* in our country.

Five *Staphylococcus* isolates identified as LinR, recovered from blood and catheter cultures of patients attending four different wards of a hospital (n=362 beds) located in the Centre of Portugal (May through November 2012), were further characterized. Epidemiological features of these isolates are described in the Table. The patients were treated with linezolid in the present (n=2) or in previous (n=2) hospitalizations, suggesting that the latter two could have been colonized with LinR strains in the moment of the first hospital discharged. *S. epidermidis* were identified by Vitek II system (bioMérieux) and antibiotic susceptibility was studied by agar dilution (linezolid, vancomycin) or disc diffusion (other 10 antibiotics; Table 1) (*5*). All isolates were screened for *cfr* gene, mutations in the 23S rRNA V domain and in genes (*rplC, rplD* and *rplV*) encoding the L3, L4 and L22 ribosomal proteins (*6,7,8*), by PCR and sequencing. Clonal relatedness was determined by Pulsed-Field-Gel-Electrophoresis (PFGE; macrorestriction with *Sma*I) and by multilocus sequence typing (MLST) (http://sepidermidis.mlst.net; http://www.cdc.gov/hai/pdfs/labsettings/ar_mras_pfge_s_aureus.pdf). In vitro adherence to abiotic surfaces was searched by a biomass quantification assay in *S. epidermidis* from patient 1 (*9*). All *S. epidermidis* were MDR, including to linezolid (MIC>32mg/L), cefoxitine, chloramphenicol, cotrimoxazol, ciprofloxacin, clindamycin and different aminoglycosides, remaining susceptible only to 4 antibiotics tested, as vancomycin (MIC = 2mg/L) (Table). To characterize LinR, our isolates were compared with the linezolid-susceptible (LinS) *S. epidermidis* RP62A/ATCC 35984 nucleotide sequence (GenBank accession no.

CP000029). They showed the previously described mutations T2530A, T2504A and G2631T, although T2504A and G2631T were also present in LinS *S. epidermidis* RP62A (*1,2*). The most common reported G2576T was not detected (*1,2*). Nucleotide mutations consistent with the amino acid changes L94V (L101V from *S. epidermidis* ATCC 12228; not related with LinR) and G152D previously described (*2,8*), as well as the new D159E and A160P in L3 ribossomal protein were observed, when comparing our isolates also with *S. epidermidis* RP62A. Mutations in this protein were linked to LinR, although definitive conclusions are not available (*8*). The *cfr* gene and mutations in ribosomal proteins L4 or L22 were not detected.

All isolates recovered during the seven months survey had the same PFGE type and belonged to ST2/CC5 (formerly in CC2) (*10*), also detected among LinR *S. epidermidis* from Europe, Brazil and USA (*1,2,3*). *S. epidermidis* from patient 1, considered representative of the observed clone, revealed a high ability to adhere to abiotic surfaces and grow in the biofilm form, feature that can facilitate infections related to indwelling medical devices. This strain was classified as strongly adherent, presenting a higher optical density (OD_{570nm} = 2.33±0.34) than blank sample (culture medium-LB+glucose; OD_{570nm} = 0.2±0.03). The OD_{570nm} of the positive control *S. epidermidis* ICE9 was 2.69±0.44. *S. epidermidis* ST2/CC5 is successfully disseminated in nosocomial setting worldwide and is characterized by a high level of genetic diversity, an increased recombination/mutation rate, biofilm production ability and a high number of SCCmec elements acquisition (*10*). In Portugal *S. epidermidis* ST2/CC5 was observed in community (*10*) but as far as we know, not yet described in the hospital setting as in the present study.

In conclusion, we reported the emergence of a methicillin and LinR *S. epidermidis* in a Portuguese hospital, persisting at least during a seven-month period. The identification of the successful MDR *S. epidermidis* ST2/CC5 clonal lineage highlight the need for strict infection control procedures along with revision of therapeutic strategies (e.g., linezolid use for the treatment of methicillin resistant *Staphylococcus* spp. only when vancomycin is not an option, due to elevated MIC or clinical failures) to preserve linezolid therapeutic effectiveness. LinR *S. epidermidis* effective control, including in patients released from the hospital and with previous therapy with linezolid is critical to prevent a potential epidemic scenario in this particular hospital and, at a larger scale, in our country, as occurred for other gram-positive as methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci.

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Table 1. Epidemiological features and antibiotic resistance characterization of linezolid resistant *Staphylococcus epidermidis* isolates recovered in a Portuguese hospital.

		Patient No.*				
		1	**2**	**3**	**4**	**5**
Epidemiological features	Date of isolation	2012 May 8	2012 Aug 7	2012 Oct 23	2012 Nov 7	2012 Nov 11
	Hospital ward	Men Surgery	Unknown	Medicine I	Emergency unit†	Emergency unit†
	Pathology	Gastric neoplasy§‡	Unknown	Multiple§	Acute lung edema	Multiple
	Clinical sample	Catheter	Blood	Catheter	Blood	Blood
	Sex/Age	Male/75	Unknown	Female/87	Male/78	Male/87
	Previous therapy with linezolid	Yes	Unknown	Yes#	Yes†	Yes†
	PFGE	A	A	A	A	A
	Sequence Type	2¶				
	Biofilm production (OD_{570nm})	Strong (2.33±0.34)ˈ				
Antibiotic resistance	Linezolid (MIC-mg/L)	R (32)	R (32)	R (32)	R (32)	R (32)
	Vancomycin (MIC-mg/L)	S (2)	S (2)	S (2)	S (2)	S (2)
	Cefoxitin	R	R	R	R	R
	Gentamicin	R	R	R	R	R
	Tobramycin	R	R	R	R	R
	Ciprofloxacin	R	R	R	R	R
	Clindamycin	R	R	R	R	R
	Erythromycin	S	I	S	I	S
	Quinupristin-dalfopristin	S	S	S	S	S
	Chloramphenicol	R	R	R	R	R
	Tetracycline	S	S	S	S	S
	Cotrimoxazol	R	R	R	R	R
	Molecular studies in linezolid resistance					
	cfr gene	-	-	-	-	-
23S rRNA mutations	T2504A	+	+	+	+	+
	G2631T	+	+	+	+	+
	T2530A	+	+	+	+	+
L3 ribosomal protein mutations	Leu94Val	+¶				
	Gly152Asp	+¶				
	Asp159Glu	+¶				
	Ala160Pro	+¶				
L4 or L22 ribosomal protein mutations		None¶				

*A sixth linezolid resistant *S. epidermidis* was detected in December 2012, although the access to this isolate was not possible during the time frame of the study.

†Patients 4 and 5 were both hospitalized in Medicine II a month before the isolation of linezolid resistant *S. epidermidis*. Therapy with linezolid was made during this first hospitalization. For patient 4, linezolid therapy was of at least 12 days. For patient 5 the duration of therapy is unknown.

#Patient 3 received 11 days of linezolid before the recover of linezolid resistant *S. epidermidis*.

[‡]Followed in oncology ward since 2011.

[§]Long stay hospitalization.

[¶]Studied in S. *epidermidis* from patient 1, representative isolate of the PFGE type A.

[ζ]For the interpretation of the results, the cutoff optical density (ODc) was defined as three standard deviations above the mean OD of the negative control (culture medium). Strains were classified as non-adherent (OD≤ODc), weakly adherent (ODc<OD≤2xODc), moderately adherent (2xODc<OD≤4xODc) or strongly adherent (4xODc<OD).

Abbreviations: R, resistant; S, susceptible; I, Intermediate resistance; MIC, minimum inhibitory concentration; +, positive; -, negative.

4.3. Anti-biofilm activity in *Staphylococcus epidermidis* and structural characterization of *Bacillus safensis* biosurfactant

Publication:

Anti-biofilm activity in *Staphylococcus epidermidis* and structural characterization of *Bacillus safensis* biosurfactant

This study, reports the prevention of biofilm formation of a mixture of pumilacidin- and surfactin-type compounds, produced by a *Bacillus safensis* strain Bs1*,* against the most frequent undwelling related pathogen, *Staphylococcus epidermidis*, including multidrug resistant strains.

Thus, our results evidence the potential of this mixture of biosurfactants towards the protection of surfaces, namely on medical devices.

Anti-biofilm activity in *Staphylococcus epidermidis* and structural characterization of *Bacillus safensis* biosurfactant

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Running title: *Bacillus safensis* biosurfactant prevents pathogenic *S. epidermidis* biofilm.

Keywords: *Bacillus safensis*, *Staphylococcus epidermidis*, pumilacidin, surfactin biosurfactant, anti-biofilm, anti-adhesive

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Abstract

Anti-adhesive or anti-biofilm properties in different bacterial species of *B. pumilus* group species biosurfactants have been reported. In this study, we evaluated the effectiveness of a biosurfactant produced by *B. safensis* Bs1, in the prevention of biofilm formation by multidrug resistant pathogenic *S. epidermidis* strains. Moreover, surface tension reduction activity, critical micelle concentration (CMC) and biochemical identification were determined. Remarkably, pre-conditioning and co-incubating experiments using Calgary Biofilm Device, with Bs1-biosurfactant significantly reduced the number of adherent cells of strong biofilm producing *S. epidermidis* isolates (>90% of inhibition) at concentrations lower than the CMC. Furthermore, our results demonstrate that the observed reduction is not a result of cell killing but instead, is the result of biofilm adhesion prevention.. LC/ESI-MS/MS analysis of lipopetides produced by *B. safensis* Bs1, revealed a mixture of pumilacidin- and surfactin-type compounds, being the variant Leu7 of pumilacidin present in a higher abundance, which showed to highly reduce the surface tension of water from 69.2 mN m^{-1} to 28.8 mN m^{-1}. A CMC value of 33.18 mg L^{-1} was also identified. In conclusion, surfactin-like compounds produced by *B. safensis* Bs1 demonstrated a relevant anti-biofilm activity that could contribute to reduce the burden of infections by *S. epidermidis,* the most frequent undwelling related pathogen.

1. Introduction

Biosurfactants are biologically produced amphiphilic compounds that reduce the surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures (Banat *et al*., 2010). Unlike their chemical counterparts, these compounds present lower toxicity, higher biodegradability and environmental compatibility, higher selectivity and specific activity at extreme temperatures, pH and salinity, as well as the ability to be synthesized from renewable feedstock (Cameotra and Makkar 1998; Banat *et al*., 2000; Banat *et al*., 2010; Kumar *et al*., 2006).

Bacterial biosurfactants were proposed to act as hydrocarbons biodegrading, emulsifiers, wetting, solubilizing, detergent and phase-dispensing agents (Fracchia *et al*., 2012; Martinotti *et al*., 2013). In addition, they revealed to be useful as antibacterial, antifungal, antiviral and anti-adhesive agents, as well as potential immunomodulatory and vaccines adjuvants compounds (Fracchia *et al*., 2012; Martinotti *et al*., 2013). Therefore, they have been considered the new generation molecules for pharmaceutical, cosmetic, health care and food industries.

Lipopetides represent one of the most predominant and efficient class of surface active agents. Surfactin family, comprising about 20 different compounds (Bonmatin *et al*., 2003), includes the best studied lipopeptides. The chemical structural characteristic of this family, with exception of esperin (Thomas and Ito 1969), is a heptapeptide possessing a chiral sequence LLDLLDL, interlinked with a β-hydroxy fatty acid and with a D-Leu in position 3 and 6 and a L-Asp in position 4 (Seydlová *et al*., 2011). In addition, amino acids residues that can be present in position 2, 4 and 7 belong to the aliphatic class and enclosing Val, Leu and Ile (Peypoux *et al*., 1991; Itokawa *et al*.,, 1994; Bonmatin *et al*., 1995). The presence of these variants provides a unique chemical structural diversity which enables a multiplicity of therapeutical applications.

Production of surfactin-like compounds has been described in some different *Bacillus* species, including in some species of *B. pumilus* group members, as *B. safensis* and *B. pumilus* (Peypoux *et al*., 1999; Fox and Bala 2000; Moran *et al*., 2000; Kalinovskaya *et al*., 2002; Branquinho *et al*., 2014b). In fact, recent structural analysis of *B. safensis* biosurfactants suggested the presence of a particular mixture containing pumilacidin and surfactin-type compounds in this species (Branquinho *et al*., 2014b).

Staphylococcus epidermidis, a human skin and mucosal commensal bacteria, is an important opportunistic pathogen being the most frequent cause of indwelling medical

device-associated infections. Its ability to cause infections is linked to its natural niche on human skin and ability to attach and form biofilm on foreign bodies (Rupp, 2014). Cells in this mode of growth are inherently more resistant to antimicrobials (Cabrera-Contreras *et al*., 2010). Additionally, often hospital isolates are methicillin-resistant *S. epidermidis* (MRSE) and multidrug resistant (MDR) (Otto, 2009), belonging to different lineages that could be successfully disseminated in nosocomial setting worldwide, like ST2 lineage (Miragaia *et al*., 2007; Gordon *et al*., 2012). Those cumulative aspects turn *S. epidermidis* infections more difficult and expensive to treat. Bacterial adhesion to surfaces is one of the initial steps that direct the microorganisms to the biofilm formation (Donlan and Costerton 2002). Therefore, treating surfaces with microbial surface-active compounds appears as an interesting strategy to control/circumvent harmful adherent cells in forms of biofilms. Surfactin class molecules have been extensively explored in numerous applications, including its involvement in reducing pathogenic microbial adhesion and its detachment from surfaces (Ongena and Jacques 2008). Although *B. pumilus* group is known as producer of surfactin-like variants (Peypoux *et al*., 1999; Fox and Bala 2000; Moran *et al*., 2000; Kalinovskaya *et al*., 2002; Branquinho *et al*., 2014b), their use as anti-adhesive/anti-biofilm agents against *S. epidermidis* is still poorly studied.

In the present study, we demonstrate the effectiveness of a biosurfactant produced by *B. safensis* Bs1 in the prevention of biofilm formation by multidrug resistant pathogenic *S. epidermidis* strains. The surface-active properties and the structural characterization of this biosurfactant are also elucidated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

2.1.1. Biosurfactant producing strain

During a study assessing the diversity of *Bacillus* spp. isolates, we identified a *B. safensis* strain designated herein as Bs1, recovered from a gastropod (terrestrial slug) normal flora collected in the Northern of Portugal (Branquinho *et al*., 2012). Isolate identification was conducted by phenotypic and genotypic (16S rRNA, *gyrB and rpoB* gene sequences) methods (Branquinho *et al*., 2014a). The bacterial isolate was maintained on Luria Bertani-Miller (LB) Agar (Oxoid, United Kingdom) for short-term storage and in LB broth supplemented with 40% (v/v) glycerol at -80 ºC for long-term storage.

2.1.2. Biofilm producing strains

Three *S. epidermidis* nosocomial strains causing infections, recovered from a catheter, wound and sputum, and a human commensal strain, all previously identified as strong biofilm producers were used in biofilm inhibition assays (Table 1). These strains, recovered in different countries (2007-2013), belong to different lineages, including the most disseminated one in nosocomial settings (Sequence type (ST) 2), present different genetic features associated with biofilm formation, and are multidrug-resistant (including a methicillin and linezolid resistant (Ste1) strain involved in a recent hospital outbreak) (Barros *et al*., 2014). All *S. epidermidis* isolates were maintained on trypticase soy agar (TSA) (Oxoid, United Kingdom) for short-term storage and in trypticase soy broth (TSB) (Oxoid, United Kingdom) supplemented with 40% (v/v) glycerol at -80 ºC for long-term storage.

2.2 Biosurfactant extraction, purification and quantification

For biosurfactant production, a seed culture was prepared by transferring a loop of *B. safensis* Bs1 strain from a LB agar overnight culture, into 10 mL of LB broth incubated for 4 h, at 28 °C and 200 rpm, on an orbital shaker. Thereafter, 2 ml of this culture were

inoculated into 500 mL of LB broth and incubated at 28 °C and 120 rpm for 24 h. A cell-free preparation from this culture was then prepared by centrifuging at 8,000xg for 30 min.

For biosurfactant extraction, the cell-free supernatant was acidified to pH 2 with HCl (6 mol L^{-1}), and left for 12 h at 4 ºC for precipitation. The precipitate was then recovered by centrifugation at 8000 g^{-1} for 10 min at 4ºC, and the resulting solution extracted twice with ethyl acetate/methanol (4:1 v/v) mixture according to the method described by Rivardo *et al*. (2009). The remaining water present in the organic phase was then removed by anhydrous sodium sulfate and finally evaporated to dryness under vacuum conditions. Acetone was added to recover raw biosurfactant, which after acetone evaporation was collected and weighted.

2.2.1. Biosurfactant stock solution and dilutions

The extracted Bs1-biosurfactant was dissolved in phosphate buffer (PBS, pH 7.2) at the final concentration of 5120 µg ml^{-1}. This solution was filtered through 0.2-µm filter (Millipore Corp., Bedford MA) and stored at 4 °C. For experiments, stock solutions of Bs1-biosurfactant were serially diluted 1:1 in PBS.

2.2.2. Surface-active properties of Bs1-biosurfactant

Screening of surface-active properties of Bs1-biosurfactant was initially conducted on the supernatant (defined accordingly conditions described in section 2.2). Oil spreading assay (Morikawa *et al*., 2000) was applied to measure the potential surface activity of the excreted biosurfactant. Briefly, 20 µl of Motor Oil 10 W-40 (Selenia, Italy) were added onto a surface of 20 mL of distilled water to form a thin layer. Subsequently, 20µl of the Bs1-supernatant were gently put onto the center of the oil layer and the diameter of the oil displacement circle formed was measured to determine the presence of biosurfactant.

Surface-active properties of the purified Bs1-biosurfactant were also evaluated. Surface tension was determined using a Sigma 703D tensiometer (KSV, NY, USA) equipped with a Du Noy platinum ring at 35 ºC, on a 20 ml of a biosurfactant solution, which was prepared in alkaline sterile demineralized water at 500 µg ml^{-1}. Distilled water was used for calibration. Critical Micelle Concentration (CMC) was then determined on serially diluted biosurfactant solutions, prepared in alkaline distilled water, and was estimated

from the intercept of two straight lines extrapolated, from the concentration-dependent and concentration-independent sections of a curve plotted between biosurfactant concentration and surface tension values.

2.3. Bacterial biofilm inhibition

2.3.1. Bacterial biofilm production

Biofilms were grown in the Calgary Biofilm device (CBD) (Innovotech Inc., Canada), commercially available as the MBEC assay system, as described by Harrison *et al*. (2006).

Cultures of the *S. epidermidis* isolates (Table 1) were firstly incubated overnight at 37 °C on TSA. Colonies were then suspended in 0.9% saline solution to obtain a 1.0 McFarland standard solution (corresponding to approximately 3.0×10^{8} cfu ml^{-1}). This suspension was diluted 30-fold in TSB (corresponding to approximately 1.0×10^{7} cfu ml^{-1}) and constituted the inoculum for the CBD. Subsequently, 150 µl of this inoculum was added to each well of the 96-well plates, the CBD peg lid fitted inside and the assembled device placed on an orbital shaker (150 rpm), which was fixed in a humidified incubator for 24 h at 37 °C. After this period, biofilms were rinsed twice to remove loosely adherent cells, by inserting the peg lids into microtiter plates prepared with 200 µl $well^{-1}$ of 0.9% saline, during 2 min.

2.3.2. Pre-coating experiments

CBD was previously coated with Bs1-biosurfactant by dipping the lid of the CBD into 200 µl of biosurfactant solutions starting from 2560 to 80 µg ml^{-1}. The CBD lid assembled with the microtiter plate was incubated at 37 °C on an orbital shaker at 125 rpm for 24 h, then removed and upside dried for 1 min before adding the correspondent *S. epidermidis* biofilm producer strains, as described in section 2.3.1. Each test was performed in triplicate on two separated biological replicates.

2.3.3. Co-incubation experiments

Co-incubation experiments were carried out in uncoated CBD. *S. epidermidis* isolates were dispensed in each well together with Bs1-biosurfactant solutions to reach concentration ranges from 5 to 150 $\mu g.ml^{-1}$ in the final volume of 200 µl. Each test was performed in triplicate on two separated biological replicates.

2.3.4. Viable cell counting

Inhibition of *S. epidermidis* biofilm formation using Bs1-biosurfactant was evaluated by biofilm viable cell-counting method. The lid of the CBD plates, treated as described in sections 2.3.2. and 2.3.3., were inserted into 200 µl of LB broth supplemented with 1% Tween 20 (Sigma-Aldrich, St. Louis, USA). Biofilms were then disrupted from the peg surface using an Aquasonic 250HT ultrasonic cleaner (VWR International, Mississauga, Canada) set at 60 Hz for 10 min. The biofilm cells were serially diluted in 0.9% saline, plated in triplicate onto TSA, which were further incubated for 24 h at 37 °C, and then colonies enumerated. Thus, anti-biofilm formation capacity of Bs1 biosurfactant was considered positive if, after 24 h of growth, there was a ≥1 $\log_{10}$ (90%) difference in the mean of CFU peg^{-1} compared to the growth control.

2.5. Antibacterial activity

To evaluate if Bs1-biosurfactant presented antibacterial effect against *S. epidermidis* isolates, well agar diffusion and broth dilution methods were used. Mueller Hinton (MH) agar cation adjusted (bioMérieux, France A) plates were swabbed with *S. epidermidis* suspension (0.5 McFarland adjusted). Purified Bs1-biosurfactant at concentrations of 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 µg mL^{-1} were then loaded into wells (5 mm diameters) punched into the agar. Distilled water and PBS were used as negative controls. Plates were incubated overnight at 37 ºC and then observed for the inhibitory zones.

In another set of experiments, the antibacterial effect of Bs1-biosurfactant extracts was detected by spectrophotometric method. Bacterial suspensions (0.5 McFarland adjusted) were incubated into 96 microtiter plates without and with Bs1-biosurfactant, at concentrations described above and analyzed spectrophotometrically at 600 nm after 24 h

of incubation at 37 ºC. Experiments were performed independently in duplicate with tree biological replicates each one.

2.6. Detection of surfactin synthethase genes

Screening for presence of the *sfp* gene, a member of the *srfA* operon required for the biosynthesis of surfactin, was carried out by PCR amplification using two set of primers. The first, *sfp*-f (5´-ATGAAGATTTACGGAATTTA-3´) and *sfp*-r (5´-TTATAAAAGCTCTTCGTACG-3´) described by Hsieh *et al.* (2008), amplified a fragment of 675 bp, enclosed in the *sfp* gene of *B. subtilis* (GenBank accession no. X63158), from position 167 to 841. PCR amplification program accomplish an initial denaturation of 94 °C for 25 s, followed by 35 cycles at 94 °C for 10 s, 46 °C at 30 s, and 72 °C for 1.5 min and by a final extension of 72 °C for 10 min.

Additionally, the detection of surfactin biosynthetic gene using degenerated primers As1 (5´-FCGCGGMTACCGVATYGAGC-3´) and Ts2-R (5´-ATBCCTTTBTWDGAATGTCCGCC-3´), described by Tapi *et al.* (2010) was also applied. PCR amplification included an initial denaturation at 94 °C for 3 min, followed by 30 cycles, with a denaturation step at 94 °C for 1 min, an annealing step of 30 s at 45 °C, followed by extension step during 45 s at 72 °C and a final extension at 72 °C for 10 min. Amplicon sequencing was achieved for identity confirmation.

Moreover, genomic DNA for PCR amplification was extracted using InstaGene^{TM} matrix (BioRad laboratories, Inc., Hercules, CA, USA) according to the manufacturer´s instructions. DNA was used directly for PCR amplification or stored at -20 ºC for further utilization.

2.7 Characterization of lipopeptides by LC/ESI-MS/MS

2.7.1 Mass spectrometry analysis

An aliquot of the Bs1 biosurfactant extract was dissolved in methanol/acetonitrile (50/50 v/v) to obtain a 1000 μg ml^{-1} stock solution. Freshly prepared working solutions were made by diluting the stock solution with methanol/water (50/50 v/v) to achieve 15 μg ml^{-1} solutions.

Mass spectrometry analyses were done on a LCQ DECA XP Plus (Thermo Finnigan, San Jose, CA, USA), Ion Trap mass instrument equipped with an ESI source. Samples (15 µg ml^{-1} solutions) were infused with a syringe at 5 µg min^{-1} flow rate. Source voltage and capillary voltage were 4.80 kV and 23 V in positive ion mode, while 5 kV and -15 V in negative ion mode. Capillary temperature and sheath gas flow (N_2) were, set respectively at 350ºC and 30 arbitrary units in both scan modes. Data were acquired in positive and negative MS total ion scan mode (mass scan range: *m*/*z* 100 2000) and in positive MS/MS product ion scan mode; the normalized collision energy (nce %) was optimized for each precursor ion selected: *m*/*z* 1030, 38%; 1044, 1058, 1072, 1086 and 1100, 39%; 1464, 1478, 1492 and 1506, 35%.

2.5.2. Liquid chromatography–mass spectrometry analysis

A Surveyor HPLC on line with a LCQ DECA XP Plus (Thermo Finnigan, San Jose, CA, USA) Ion Trap mass spectrometer equipped with an ESI source was employed. Separations were performed on an analytical Luna 5 µm C18, 150×4.6mm (Phenomenex, Torrance, CA) protected with a C18-Security Guard cartridge, 4 × 3.0 mm (Phenomenex). The injection volume applied was 10 µl. Mobile phase components encompassed: A - formic acid/ ammonium formate buffer and B- acetonitrile. Moreover, lipopeptides were eluted according the following gradients: gradient 1 A: B (60: 40) for 4 min, then A: B (0: 100)over 24 min and finally 100% B over 6 min, at a flow rate of 0.8 ml min^{-1} ; gradient 2 A: B (20: 80) for 5 min, then A: B (0: 100) over 20 min and finally 100% B over 15 min, at a flow rate of 0.8 ml min^{-} LC/MS full scan positive mode was performed from *m*/*z* 100 to 2000. Alternatively, LC/ESI-MS/MS modalities were applied to the selection of precursor ions, following the conditions set during the infusion analysis.

2.8. Interpretation of results and statistical analysis

The efficiency of biofilm inhibition was assessed by determining the minimal biofilm eradication concentration (MBEC) after 24 h, by viable cell count. The effect of Bs1-biosurfactant was expressed as $log^{10}CFU\ mL^{-1}$ ± SD.

Statistical analysis was performed using GraphPad Prism version 6.00 software (GraphPad, Sandiego, USA). Data were analyzed by ANOVA test and means were

compared using Kruskal-Wallis test with P value of 0.05. Treatments were considered significantly different if P≤0.05.

3. Results and Discussion

3.1. Surface-active properties of Bs1-biosurfactant

The production and chemical composition of biosurfactants depends among other factors, on the culture medium in which the producer's strains are cultivated. Nevertheless, and since LB broth has been extensively used for biosurfactant production from *Bacillus* strains (Thaniyavarn *et al*., 2003; Rivardo *et al*., 2009), it was selected for the evaluation of *B. safensis* biosurfactant production.

Initial screening of surface tension (ST) properties conducted on the BS1-supernatant by oil spreading assay revealed a clearly formed oil displacement circle of 20 mm. Additionally, surface tension of Bs1-supernatant, determined using a Du-Noy Tensiometer, revealed a ST value of 33.2 mN m^{-1} ±0.35 compared with 69.2 mN m^{-1} ±0.12 of water, indicating the presence of a compound with a remarkably tensioactive property.

Moreover, analyses of ST properties of the purified Bs1-biosurfactant revealed consistency with previous Bs1-supernatant ST results. Figure 1 shows ST values of the purified Bs1-biosurfactant at different concentrations. Bs1-biosurfactant exhibited an excellent surface tension reducing activity, decreasing the surface tension of water from 69.2 mN m^{-1} to 28.8 mN m^{-1}, at concentrations up to 500 µg mL^{-1}. Then values slowly increased to 20 µg mL^{-1}, concentration from which a sharp increment of surface tension was observed.

This high efficiency property is similar to that reported for surfactin molecule, with a value of 27 mN m^{-1} (Mulligan, 2005).

The critical micelle concentration (CMC) is an index to evaluate surface activity. By definition, CMC is the surfactant concentration above which micelles are spontaneously formed. Until the CMC is reached a decrease in the surface tension will be observed. Nevertheless, upon reaching the CMC, any further increase in the surfactant concentration will only increase the number of micelles and no alteration in the surface tension will be observed (Sobrinho *et al*., 2008). Critical micelle concentration determined for BS1-biosurfactant was 33.18 mg l^{-1}.

The Bs1-biosurfactant CMC value obtained is in agreement with those presented in the literature for other surfactin-like compounds. For instance, in the case of *B. subtilis*

surfactin producers strains Abdel-Mawgoud *et al*. (2008) characterized a surfactin-like compound produced by a *B. subtilis* BS5 isolate with a CMC value of 15.6 mg l^{-1}, whereas other examples suggested values of 11 mg l^{-1} (Nitschke and Pastore, 2006; Mulligan, 2005; Barros *et al*., 2008), 13, 17 and 22 mg l^{-1} (Ramkrishna and Swaminathan, 2004) and 40 mg l^{-1} (Vaz *et al*., 2012), for similar compounds. In addition, for *B. pumilus* producer's strains, Slivinski *et al.* (2012) reported that *B. pumilus* UFPEDA 448 isolate was able to produce a ST reducing compound, which presented a CMC of 12.2 mg l^{-1}. Contrastingly, Oliveira and Garcia-Cruz (2013) using vinasse, a byproduct of ethanol production, and a waste frying oil from vinasse as substrates for *B. pumilus* biosurfactant production, reported CMCs values for these biosurfactants, respectively of 1500 and 200 mg l^{-1}. Actually, the differences observed in these reported values may be attributed to a lower concentration of the surfactin-like compounds due to the low degree of purification, or even to the presence of a different variant.

Moreover, organoleptic characteristics such as the peculiar bad odor observed for Bs1-biosurfactant were also reported by other authors in *Bacillus*-derived biosurfactants (Abdel-Mawgoud *et al*., 2008; Rivardo *et al*., 2009).

3.2. Capacity to inhibit or reduce biofilm formation of *S. epidermidis* isolates

Biofilms are source of infections of many pathogenic microbes, *Staphylococcus epidermidis* is the most frequent cause of device-associated infections, because it is known to cause biofilms that grow on catheters or other surgical implants (Rupp, 2014) In these set of experiments, the potential of Bs1-biosurfactant to inhibit biofilm formation of four biofilm-producing MDR *S. epidermidis* strains and including a representative of a linezolid resistant strain causing a recent nosocomial outbreak (Barros *et al*., 2014), was evaluated.

The effect of pre-coating polystyrene surfaces with Bs1-biosurfactant on *S. epidermidis* isolates are shown in figure 2. Interestingly, results revealed that at low concentrations, Bs1-biosurfactant was able to reduce the biofilm formation of the four *S. epidermidis* strains. Indeed, it was observed that the concentrations ranging between 6.25 µg ml^{-1} and 100 µg ml^{-1} were the most effective in preventing the biofilm formation of *S. epidermidis* isolates. In particular, in the presence of such concentrations *S. epidermidis* 55 biofilm was inhibited by >95.8% (Figure 2b), Ste1 by >93.7% (Figure 2a), Taw113 by >91.9% (Figure 2c) and Ice120 by >89.1% (Figure 2d), during 24 h of incubation.

Statistical analysis carried on the biofilm viable cell counts values demonstrated that there is a significant activity of Bs1-biosurfactant at these concentrations ($p<0.05$). Moreover, the increase of Bs1-biosurfactant concentration to 1280 and 2560 µg ml^{-1} did not significantly affect biofilm formation of multidrug resistant *S. epidermidis* isolates, namely, LinR, Taw 113 and Ice 120 (Figure 2 a, c and d), whereas biofilm of *S. epidermidis* 55 isolate remains significantly affected (Figure 2 b).

The effect of co-incubation of *S. epidermidis* biofilm producer strains and Bs1-biosurfactant is shown in figure 3. Low concentrations of Bs1-biosurfactant (10-20 µg ml^{-1}) revealed ability to reduce the biofilm formation of the four *S. epidermidis* strains. Biofilm formation of *S. epidermidis* Ste1 and 55 were reduced by 92.1% and 92.5% respectively, applying 10 µg ml^{-1} of Bs1-biosurfactant; Taw113 by 96.7% and Ice120 by 87.9%, applying 20 µg ml^{-1} of Bs1-biosurfactant.

Statistical analysis carried out on the biofilm viable cell count values showed a significant activity of Bs1-biosurfcatant at these concentrations ($p<0.05$). Moreover, concentrations higher than 100 µg ml^{-1} did not significantly reduced the adherence of all *S. epidermidis* strains.

Overall, considering results from pre-coating and co-incubation experiments our data corroborate previous observations by Rivardo *et al.* (2009), where an increase in biosurfactant concentration did not affect significantly biofilms formation or even its reduction. Moreover, other studies reported that high concentrations of biosurfactant can stimulate the cell growth, as they could be used as source of nutrients (Fowler *et al*., 2008). In fact, Fowler *et al.* (2008) suggested that the adherence of *S. aureus* biofilms could be increased in the presence of certain concentrations of cyclic lipopetides, as they are analogous of quorum sensing auto-inducers, which stimulates the adhesion of these pathogens on surfaces.

Beyond the observation that Bs1 displays an anti-biofilm effect, its ability to interfere in the adherence process of *S. epidermidis* pathogens to polystyrene surfaces can be also established. The process involved in the adherence of a bacterium to a surface, during the first few hours, is reversible (Marshall, 1994; Høiby *et al*., 2001). Hence, preventing bacterial adhesion at the preliminary stage of adherence can reduce the risk of biofilm formation. Therefore, since we have noticed that pre-coating and co-incubating surfaces with Bs1-biosurfactants reduced the number of *S. epidermidis* viable cells adhered to the pegs, this observation prompt us to suggest that this compound can also act as an anti-adhesive molecule.

3.3. Antibacterial activity

In parallel, the effect of Bs1-biosurfactant on *S. epidermidis* cells was also tested. In the well diffusion assay, no inhibition zones were observed at all concentrations tested, after 24 h of incubation. Similarly, in the broth dilution method, no significant differences were observed between the OD values of controls and the treated cells at every concentration tested after 24 h of incubation, witha ΔOD<0.69±0.039 for strain 55, <0.97±0.05 for Ste1, <0.90±0.04 for ICE120 and of <0.77±0.04 for Taw113, suggesting that there was no apparent effect on *S. epidermidis* viability by the presence of Bs1-biosurfactant.

3.4. Detection of surfactin synthethase genes by PCR

Several genetic studies were undertaken to identify the genes required for the production of surfactin. One of the genes involved in surfactin biosynthesis is a large operon of 25kb, named *srfA*, which is also responsible for sporulation and competence development. The *sfp* gene was mapped 4kb downstream of the *srfA* operon and was associated with but not integrated in the latter, and was reported as the second gene essential for the production of surfactin. Its nucleotide sequence was established by Nakano *et al*. (1992) and the encoded enzyme, termed Sfp, belongs to the superfamily of 4´-phosphopantetheinyl transferase, which comprises the surfactin synthetase complex.

Using the primers described by Hsieh *et al*. (2004), *sfp* gene could not be detected in Bs1 isolate. Cluster analyses of different *sfp* genes available in GenBank database, including 4´-phosphopantetheinyl transferase from *B. pumilus* SAFR-032 and ATCC 7061 and others from *B. subtilis,* revealed a high level of dissimilarities between these structures, which can justify the fact that no amplicon was detected using this primers pair. Thus, *B. safensis* Bs1 possibly has a mutant allele of *sfp* that could not be detected by this pair of primers or has a slightly different homologue.

Nevertheless, using the surfactin degenerated primers described by Tapi *et al*. (2010) for the biosynthetic gene of surfactin, an amplicon with the expected length was obtained. Blast comparisons results revealed a 75% of homology with gene *srfAB* of *Bacillus amyloliquefaciens* FZB42 (GenBank accession number CP000560.1), which encode for the surfactin synthetase II, suggesting the presence of a different homologue from the described surfactin gene and that possibly a surfactin-like compound may be involved in the reported tensioactive property. The expression of different homologues or different

ratios of the same homologue are common features described in the literature for *B. subtilis* group member's biosurfactant producers. Using, LC/ESI-MS/MS characterization of Bs1 biosurfactant we confirmed the presence of a surfactin-like analogue produced by *B. safensis* Bs1, which can justify the level of homology found for the presented gene.

3.5. Bs1 characterization and structure elucidation by LC/ESI-MS/MS

Related to the *B. safensis* Bs1 characterization, full-scan mass spectra (Figure 4) through ESI-MS direct infusion analysis, operating in positive full scan mode, showed one cluster of peaks with 14 Da difference on its molecular ion species, which in accordance with other work performed by our research team (Branquinho *et al*., 2014b), correspond with spectral analysis of pumilacidin variants Leu7 and Val7, with a relative abundance average of 83 and 7%, respectively for each one (Table 2). Four main signals at 1058, 1072, 1086 and 1100 *m/z* were detected, which correspond to the sodiated molecules $[M+Na]^+$. Therefore, the positive LC-ESI-MS/MS analysis on the sodiated molecule at *m/z* of 1058, 1072, 1086 and 1100, revealed that these signals were in according with the four homologues of pumilacidin compounds, respectively C-14, C-15, C-16 and C-17 Leu7 variants and also C-15 and C-17 Val7 pumilacidin variant (Branquinho *et al*., 2014b). Moreover, our results are also in accordance with the presence of two surfactin homologues, e.g. C15- Glu/Leu/Leu/Val/Asp/Leu/Leu and C7- Glu/Leu/Leu/Val/Asp/Leu/Leu (m/z of 1058 and 1086), with a relative abundance average of 10% (Table 2).

Thus, these results demonstrate that a mixture of pumilacidin and surfactin molecules produced by *B. safensis* interfered in *S. epidermidis* adhesion ability and consequently, on its biofilm formation capability to polystyrene surfaces, but did not inhibit the growth of these pathogens, despite the previous reports of an antibacterial activity of lipopeptides belonging to surfactin family (Compaoré *et al*., 2013). In addition, since Bs1-biosurfactant interferes with bacterial adhesion, this could be of great benefit as it could improve bacterial clearance by the host immune system.

S. epidermidis is one of the most prevalent causes of nosocomial infections, and is often associated with infections caused by indwelling medical devices, namely due to its ability to produce biofilms, which seriously compromise the success of human therapy. Moreover, clinical *S. epidermidis* strains present a high rate of antibiotic resistance to methicillin (70%) and its overwhelming majority is also resistant to other antimicrobial

classes (Mendes *et al*., 2012). Nevertheless, among this species, low rates of resistance to linezolid have been described, and, in Portugal, just one outbreak of a clinical methicillin and linezolid resistant *S. epidermidis* (isolate Ste1, included in this study) was reported (Mendes *et al*., 2012; Barros *et al*., 2014). Thus, the use of an anti-adhesive/anti-biofilm compound, with remarkably potential capacity to circumvent infection caused by MDR biofilm producers' bacteria, is of great interest. In fact, biofilm formation is a complex phenomenon that is usually divided into five steps: reversible adhesion, EPS production, maturation and dispersion. The first and second steps involve microbial adhesion to surfaces and are the most important to the initiation of biofilm formation. These steps involve physico-chemical interactions that can be mediated by non- specific interactions with long-range forces, including Lifshitz-van der Walls interactions, electrostatic, acid-base interactions and Brownian motion forces. In addition, the hydrophobic interactions between the abiotic surface and the microorganism have a major role in the initial microbial adhesion and, therefore, biofilm development in biological systems (Chae *et al*., 2006; Goulter *et al*., 2009). Therefore, compromising this two first steps, by preventing or inhibiting bacterial adherence, with the application of a biosurfactant compound, can successfully avoid the spread of bacterial infections and also to produce a better clinical outcome, since without the formation of biofilm matrix there will be possible an improvement of antibiotic penetration.

The biofilm inhibitory effect of a biosurfactant seems to be dependent on diverse factors such as type of compound, target microorganism and the surface properties (Walencka *et al*., 2008). In fact, biosurfactants penetrate into the interface between the solid substrate and the biofilm, adsorbing at the interface and reducing the interfacial tension. Then, attractive interactions between the bacterial surfaces and solid surfaces may be decreased, which would easily lead to the removal of the biofilm. Rodrigues *et al*. (2006) suggested that biosurfactants reduce hydrophobic interactions, resulting in a decrease in the hydrophobicity of the surface, which interferes with the microbial adhesion to the surface and consequently, alters biofilm development. In fact, hydrophobic surfaces have shown to be particularly colonized by microorganisms, probably because these surfaces can promote the contact between microorganism and solid substratum, favoring the elimination of interfacial water present in the interacting surfaces (Rodrigues *et al*., 2006). Therefore, when a surface is conditioned with a biosurfactant, it becomes more hydrophilic, leading to a decrease of microbial attachment. Thus, and considering that surfactin has an anionic nature due to aspartic and glutamic acid residues, which are negatively charged at neutral pH (Shen *et al*., 2011), the observed anti-biofilm formation

by Bs1 biosurfactant, can be due to the fact that this compound turns the polystyrene less hydrophobic, probably as consequence of its orientation on the surface.

In the scientific literature, some reports have demonstrated the interest of surfactin-like molecules in the adhesion inhibition of pathogenic bacteria. Rivardo *et al.* (2009) showed that a biosurfactant containing surfactin and fengycin selectively inhibited biofilm formation of two pathogenic strains – *S. aureus* and *Escherichia coli* by 97% and 90%, respectively, on polystyrene surfaces but did not inhibit *S. epidermidis* biofilm producers. Additionally, other works showed that pre-coating of stainless steel and polypropylene surfaces with 0.1% (w/v) surfactin reduced the number of adherent cells of *L. monocytogenes* and *Chronobacter* and the adsorption of surfactin on polystyrene surfaces reduced the colonization of *L. monocytogenes, S. aureus, Salmonella enteritidis* and *Micrococcus luteus* (Nitschke *et al*., 2009; Zeraik and Nitschke, 2010; Gomes and Nitschke, 2012).

Thus, our results demonstrate an efficient reduction (>90%) in biofilm adherence and formation of multidrug resistant *S. epidermidis* isolates belonging to different lineages causing infection, applying a mixture of surfactin-like biosurfactants, comprising mainly pumilacidin variant Leu7, produced by a *B. safensis* strain. Despite the *B. pumilus* and *B. safensis* being considered attractive sources of biotechnological and industrial commercial products, *B. safensis* biosurfactants are not yet explored, been the present work the first evidence of its application.

4. Conclusions

Our results demonstrate the potential of Bs1-biosurfactant towards the protection of surfaces, namely medical devices, from *S. epidermidis* contamination.

Further investigations need to be done for a better comprehension of the mechanisms involved in *S. epidermidis* biofilm formation inhibition of Bs1-biosurfactant, including the effect of pH, ion force, temperature and nutrients in the hydrophobicity of the surfaces pre-conditioned with this biosurfactant.

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Table 1. Biofilm producing *Staphylococccus epidermidis.*

S. epidermidis isolate	Country	Origin	Biofilm forming capacity[a]	Biofilm genes	Sequence Type	Antibiotic Resistance profile	Reference
Ste1	Portugal	Medical device(catheter)	++	ND[b]	2	MRSE[d] LZD-R[e]	Barros *et al*., 2014
55	Italy	Skin	++	*ica*, *aap*, *atlE*	NA[c]	MRSE	Cavallo, 2011
Taw113	Taiwan	Sputum	++	*aap*	85	MRSE	Miragaia *et al*., 2007
Ice120	Iceland	Wound	++	*ica*, *aap*	89	MRSE	Miragaia *et al*., 2007

[a]Biofilm forming capacity previously tested by the microtiter assay using crystal violet.

[b]ND – not detected.

[c]NA – not applicable.

[d]MRSE - Methicillin-resistant *S. epidermidis* nosocomial outbreak isolates.

[e]LZD-R - Linezolid (MIC>32mg/L) resistant *S. epidermidis.*

Table 2. LC/ESI-MS/MS analysis of pumilacidin and surfactin homologues produced by *Bacillus safensis* Bs1 strain.

Isolate	Pumilacidin-type								Surfactin-type			
	Pumilacidin Leu7				Pumilacidin Val7							
	MW	Leu2/Leu4/Leu7	M+Na]$^+$ *m/z*	Relative %	MW	Leu2/Leu4/Val7	M+Na]$^+$ *m/z*	Relative %	MW	Leu2/Val4/Leu7	M+Na]$^+$ *m/z*	Relative %
Bs1	1035	C14-Leu2/Leu4/Leu7	1058	83	1035	C15-Leu2/Leu4/Val7	1058	7	1035	C15-Leu2/Val4/Leu7	1058	10
	1049	C15-Leu2/Leu4/Leu7	1072									
	1063	C16-Leu2/Leu4/Leu7	1086		1063	C17-Leu2/Leu4/Val7	1086		1063	C17-Leu2/Val4/Leu7	1086	
	1077	C17-Leu2/Leu4/Leu7	1100									

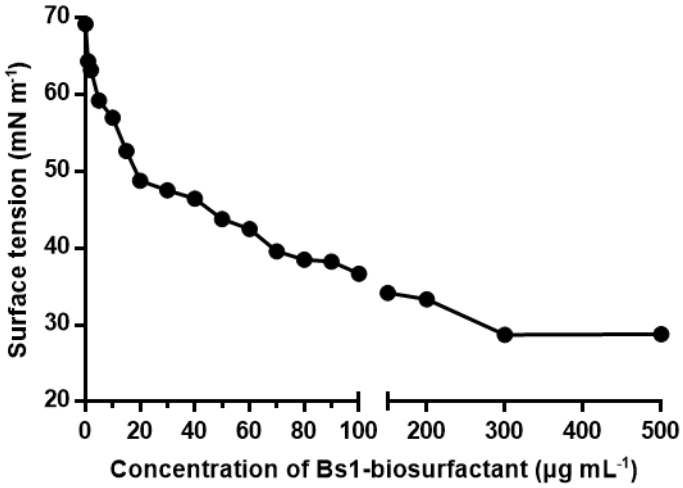

Figure 1. A plot of surface tension as a function of concentration of purified Bs1-biosurfactant. Standard deviation ranged between +/- 0.3 mN m^{-1}.

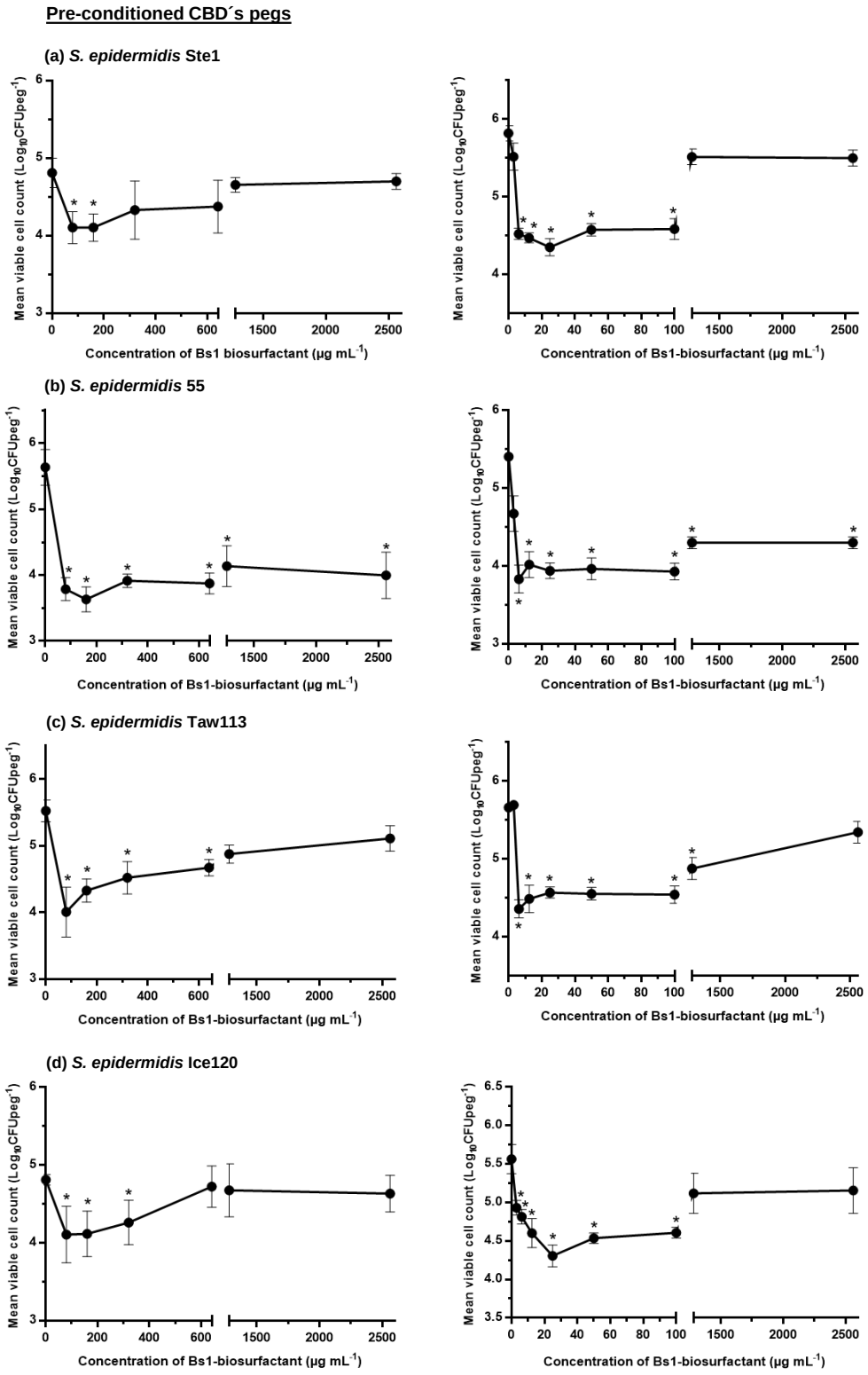

Figure 2. Effect of different concentrations of Bs1-biosurfactant on the adhesion of *S. epidermidis* Ste1 (a), *S. epidermidis* 55 (b), *S. epidermidis* Taw113 (c), and *S. epidermidis* Ice120 (d) biofilm producers, using pre-conditioned CBD´s pegs.

* indicates a significant ($p<0.05$) difference in the biofilm viable cell count at the correspondent concentration, when compared with control (without biosurfactant).

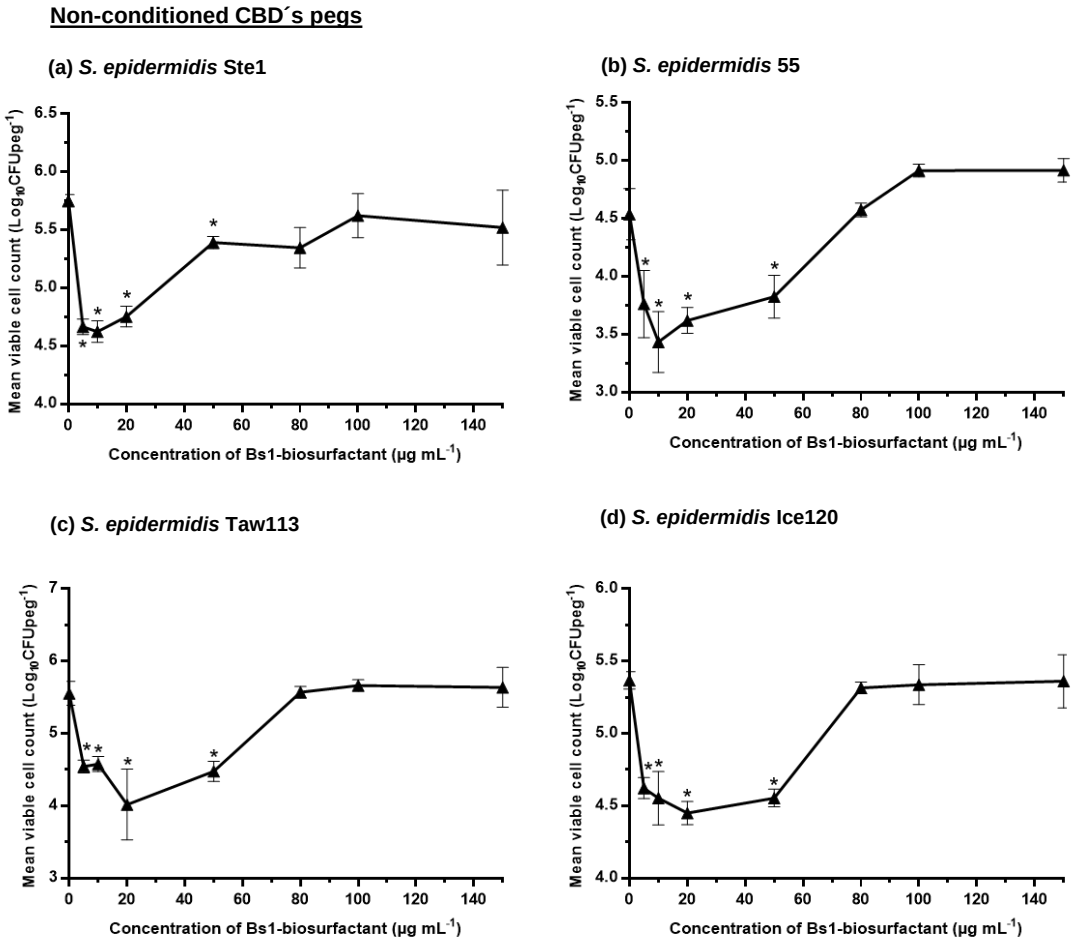

Figure 3. Effect of different concentrations of Bs1-biosurfactant non-conditioning CBD´s pegs (biosurfactant co-incubation), on the adhesion of *S. epidermidis* Ste1 (a), *S. epidermidis* 55 (b), *S. epidermidis* Taw113 (c), and *S. epidermidis* Ice120 (d) biofilm producers.

* indicates a significant ($p<0.05$) difference in the biofilm viable cell count at the correspondent concentration, when compared with control (without biosurfactant).

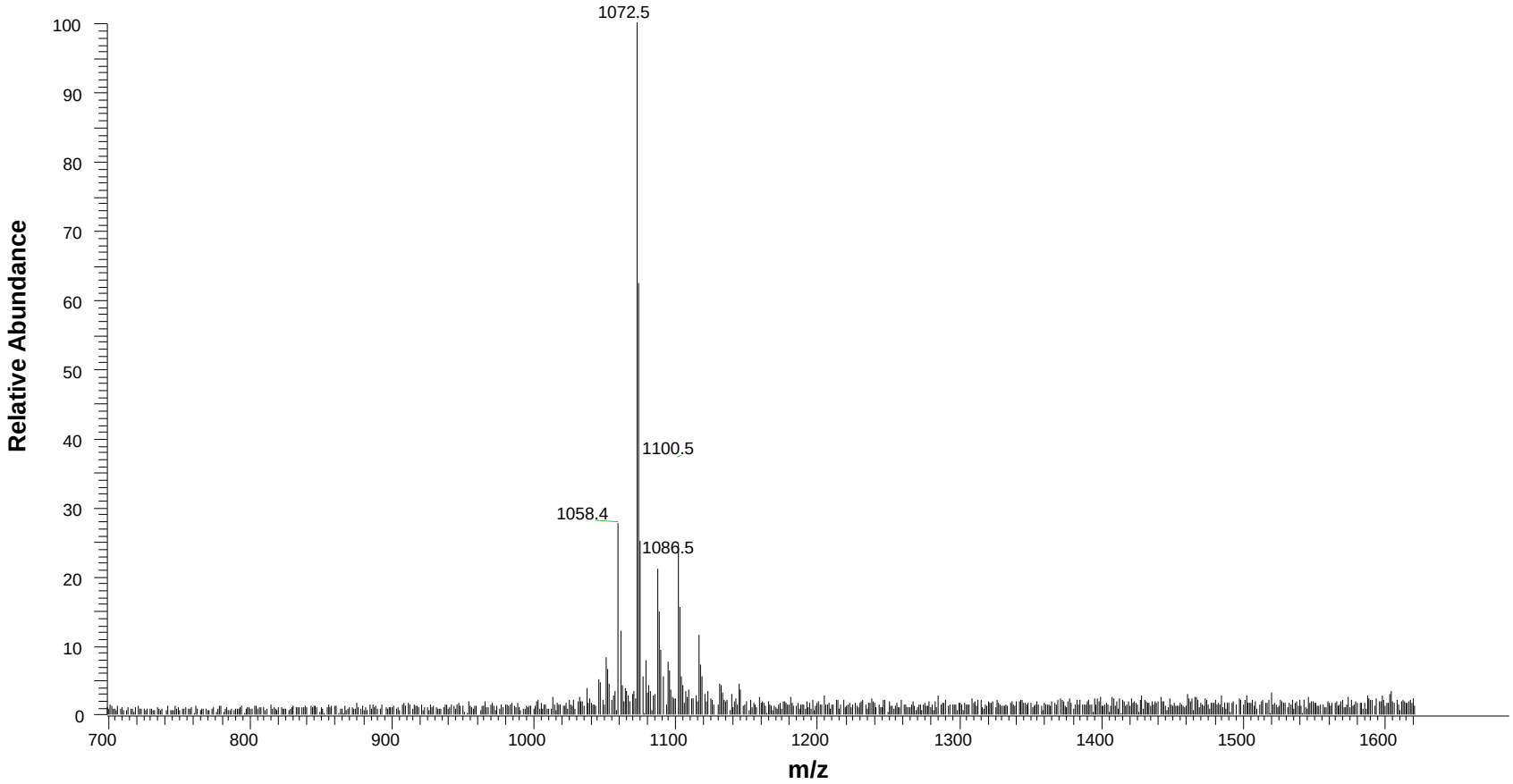

Figure 4. Full-scan mass spectra of Bs1 extract through (+) ESI-MS direct infusion analysis. Cluster of pumilacidin and surfactin are shown in *m/z* 1000-1150.

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CHAPTER 5

GENERAL CONCLUSIONS

If we knew what it was we were doing, it would not be called research, would it?

(Albert Einstein)

The overall results of this thesis indicate that *Bacillus safensis* Bs1 is an *Arion ater* adapted clonal lineage with antimicrobial activities of potential interest for the pharmaceutical and/or food industry sectors. Moreover, and beyond the contribution to accurately discriminate *B. pumilus* group species, the present work also demonstrates their genomic plasticity, which can justify their widespread into different environments and, consequently, its ability to produce a diverse array of distinct compounds with a broad range of activities.

This general statement is based on the conclusions established after the accomplishment of the specific aims. They can be summarized as follows:

1. Diversity and phylogeny within *B. pumilus* group species

- **Delineation of *B. pumilus* group species should be supported on phylogenetic trees based on concatenated housekeeping genes**, due to the high similarity between *gyrB* and *rpoB* gene sequences that prevents correct species assignments only based on sequences homology.
- **Phyogenetic tree analysis, based on *gyrB* and *rpoB* gene sequences, determined the reclassification of the medicine-slug strain as *B. safensis*** (Bs1) as well as **most *B. pumilus* isolates** from multiple origins, which were assigned to either *B. safensis* or *B. altitudinis* closed species. Moreover, this characterization extended *B. safensis* recognized habitats and unveiled their biotechnological significance.
- A **new species within *B. pumilus* group was recognized**, which representative isolate was recovered from a health's product contaminant from Portugal, and for which the name *Bacillus invictus* sp. nov. is proposed.
- ***B. pumilus* group species comprises a clonally diverse population**, which can justify their great adaptation to different niches, and the variety of bioactive compounds produced.
- Evidence of **a clonal host specificity of *B. safensis* Bs1**, suggests an adaptative process leading to the production of characteristic bioactive compounds that enables its survival to gut-slug conditions.
- **Characteristic fingerprints of *B. pumilus* and *B. safensis* obtained by MALDI-TOF MS and FTIR supports their phylogenetic assignment** and demonstrate the suitability

of these techniques to rapidly and at low cost discriminate bacterial species from *B. pumilus* group. Moreover, using MALDI-TOF MS analyses **ribosomal or spore's proteins were tentatively assigned as specific biomarkers of *B. pumilus* and *B. safensis***.

2. Antimicrobial susceptibility and virulence within *B. pumilus* group species

Microbiological antibiotic resistance to main antibiotic classes and virulence (entero-, emetic- and cyto-toxins) genes were absent, as required for their usage in the food sector. Nevertheless, from a clinical perspective non-susceptibility to cefotaxime was observed in all *B. pumilus* group isolates, which would lead to an empirical therapeutic failure with this commonly used antibiotic. Therefore, susceptibility testing to this antibiotic should be consider for granting a Qualitative Presumption of Safety status for a *Bacillus* spp. strain intended to use in the food sector.

3. Peptides with antimicrobial activity of *B. pumilus* group species

- **Characterization of the antimicrobial peptides with anti-staphylococci activity** in *B. safensis* strain Bs1 revealed the presence of a cationic peptide comprising 48 amino acid residues, which was not previously recognized as anti-bacterial. Further studies will elucidate its conformational structure, spectrum of activity, mode of action, toxicity and therapeutic efficacy in bacterial infections, relevant properties for a potential industrial application.
- **Most *B. pumilus* and *B. safensis* isolates were able to produce biosurfactants, although they were absent in *B. altitudinis***. Interestingly, a **species-specific composition** was detected, with a mixture of pumilacidin variants and surfactin compounds in *B. safensis*, whereas in *B. pumilus* only pumilacidin variants were identified.

- **A surfactin-like biosurfactant with strong anti-adhesive** activity against biofilm producing *Staphylococcus epidermidis* isolates was produced by Bs1 strain, which did not result from planktonic bacterial growth inhibition. This activity was for the first time associated with a mixture of pumilacidin- and surfactin-type variants that could contribute to reduce the burden of infections by *S. epidermidis,* the most frequent indwelling medical devices related pathogen.

ANNEXES

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Table A1. *Bacillus pumilus* group members isolates (n=41) (*B. pumilus*, *B. safensis* and *B. altitudinis*) characterized in this thesis and correspondent assigned GenBank accession numbers.

Species	Isolate	Origin/Product	Year/ Location	GenBank accession numbers			References
				16S rDNA gene	*gyrB* gene	*rpoB* gene	
B. pumilus	Bp ATCC14884			JF749284	KC895462	KC895450	Reference strain
	Bp ATCC7061^{T}			EU138517	AY167869	AB353945	Type-strain
	Bp7	Healths´s contaminants (n=3)	2005/Portugal[1]	JN699029	JX183193	JX183164	Branquinho *et al.,* 2012
	Bp11		2005/Portugal[1]	JN699027	JX183196	JX183167	
	Bp15		2005/Portugal[1]	JN699025	JX183200	JX183171	
B. safensis	BsFO-36b^{T}	Clean-room/ air particulate (n=1)	1999/USA[6]	AF234854	AY167867	KC895451	Satomi *et al.,* 2006
	Bs32	Clean-room/entrance floor (n=1)	2001/USA[6]	AY167886	AY167877	KC895453	
	Bs42	Clean-room/anteroom (n=1)	2001/USA[6]	AY167884	AY167876	KC895454	
	Bs34	Clean-room/floor (n=2)	2001/USA[6]	AY167881	AY167873	KC895455	
	Bs35		2001/USA[6]	AY167880	AY167872	KC895456	
	Bs36	Clean-room/cabinet top (n=1)	2001/USA[6]	AY030327	AY167878	KC895452	
	Bs37	Clean-room/Mars Odyssey spacecraft surface (n=5)	2001/USA[6]	AF526907	KC895457	KC895445	
	Bs38		2001/USA[6]	AF526902	KC895458	KC895446	
	Bs39		2001/USA[6]	AF526898	KC895459	KC895447	
	Bs40		2001/USA[6]	AF526896	KC895460	KC895448	
	Bs41		2001/USA[6]	AF526895	KC895461	KC895449	
	Bs1	Animals Gastropods (n=3)	2004/Portugal[1]	JN699032	JX183188	JX183159	Branquinho *et al.,* 2012
	Bs2		2005/Portugal[1]	JX183146	JX183189	JX183160	
	Bs3		2007/Portugal[1]	JN699031	JX183190	JX183161	
	Bs13	Healths´s contaminants (n=3)	2005/Portugal[1]	JX183149	JX183198	JX183169	

	Bs16		2005/Portugal[1]	JN699024	JX183201	JX183172	
	Bs17		2005/Portugal[1]	JX183151	JX183202	JX183173	
	Bs5	Cosmetic´s contaminants (n=4)	2002/Portugal[1]	JN699030	JX183191	JX183162	
	Bs18		2002/Portugal[1]	JN699023	JX183203	JX183174	
	Bs19		2002/Portugal[1]	JN699022	JX183204	JX183175	
	Bs27		2002/Portugal[1]	JN699021	JX183212	JX183183	
	Bs24	Food´s contaminants/salame (n=3)	2004/Italy[3]	JX183156	JX183209	JX183180	Matarante *et al.,* 2004
	Bs25		2004/Italy[3]	JX183157	JX183210	JX183181	
	Bs33		2004/Italy[3]	KC895444	KC895463	JX183187	
	Bs22	Plant Growth Promoters (PGPR) (n=2)	1997/USA[4]	JX183154	JX183207	JX183178	Jetiyanon 1997
	Bs23		1997/USA[4]	JX183155	JX183208	JX183179	
	Bs31	Food/beans (n=1)	2003/Africa[5]	KC895443	JX183215	JX183186	Ouoba *et al.,* 2004
B. altitudinis	Ba6	Healths´s contaminants (n=5)	2005/Portugal[1]	JX183147	JX183192	JX183163	Branquinho *et al.,* 2012
	Ba8		2005/Portugal[1]	JN699028	JX183194	JX183165	
	Ba9		2005/Portugal[1]	JX183148	JX183195	JX183166	
	Ba12		2005/Portugal[1]	JN699026	JX183197	JX183168	
	Ba14		2005/Portugal[1]	JX183150	JX183199	JX183170	
	Ba26	Food´s contaminants/rice (n=1)	2006/Norway[2]	JX183158	JX183211	JX183182	From *et al.,* 2007
	Ba20	Plant Growth Promoters (PGPR) (n=2)	1996/USA[4]	JX183152	JX183205	JX183176	Wei *et al.,* 1996
	Ba21		1997/USA[4]	JX183153	JX183206	JX183177	Jetiyanon 1997
	Ba30	Food/beans (n=1)	2003/Africa[5]	KF060662	JX183214	JX183185	Ouoba *et al.,* 2004

Table A2. Control, reference and type strains used in this thesis.

Species/Strain	Source	Control/Applicability	Reference
Gram positive			
Bacillus pumilus ATCC 7061	American Type Culture Collection (ATCC)	Phylogenetic analysis; FTIR-ATR and MALDI-TOF/MS discrimination control	Type Strain
Bacillus pumilus ATCC 14884	American Type Culture Collection (ATCC)	Phylogenetic analysis; MALDI-TOF/MS discrimination control	Reference Strain
Bacillus pumilus SAFR-032	Spacecraft Assembly Facility - NASA Jet Propulsion Laboratory – USA (2007)	Phylogenetic analysis	Gioia *et al*., 2007
Bacillus safensis FO36-b	Spacecraft Assembly Facility - NASA Jet Propulsion Laboratory – USA (1999)	Phylogenetic analysis; FTIR-ATR and MALDI-TOF/MS discrimination control	Type Strain; Satomi *et al*., 2006
Bacillus altitudinis Ba41KF2bT	High-elevation air sample – India (2006)	Phylogenetic analysis	Type Strain; Shivaji *et al*., 2006
Bacillus subtilis Bsb28	Medicine´s contaminants – Portugal (2005)	Biosurfactant producer testing	
Bacillus subtilis Bsb_{FFUP1}	Faculty of Pharmacy of Porto University Culture collection (FFUPCC)	Antimicrobial susceptibility testing	Branquinho *et al*., 2014b
Bacillus subtilis Bsb_{BGA}	Spore suspension	Antimicrobial susceptibility testing	Merck, Darmstadt, Germany – Product number 1106490001
Bacillus cereus$_{FFUP1}$	Faculty of Pharmacy of Porto University Culture collection (FFUPCC)	Toxin testing control strain	Branquinho *et al*., 2014b
Lysinibacillus fusiformis	Faculty of Pharmacy of Porto University Culture collection (FFUPCC)	Antimicrobial susceptibility testing	Branquinho *et al*., 2007
Staphylococcus aureus ATCC 29213	American Type Culture Collection (ATCC)	Antimicrobial susceptibility testing control strain	Reference Strain
Staphylococcus aureus methicillin resistant (MRSA) FFUP 001	Faculty of Pharmacy of Porto University Culture collection (FFUPCC)	Antimicrobial susceptibility testing	

Staphylococcus epidermidis linezolid resistant Ste1	Medical device(catheter) – Portugal (2013)	Biofilm producer control strain; Anti-adhesive testing activity	Barros *et al*., 2014
Staphylococcus epidermidis 55	Human skin – Italy (2011)	Biofilm producer control strain; Anti-adhesive testing activity	Cavallo, 2011
Staphylococcus epidermidis Taw113	Sputum – Taiwan (2007)	Biofilm producer control strain; Anti-adhesive testing activity	Miragaia *et al*., 2007
Staphylococcus epidermidis Ice120	Wound - Iceland (2007)	Biofilm producer control strain; Anti-adhesive testing activity	Miragaia *et al*., 2007
Enterococcus faecium vancomycin resistant (VRE) E1	Portuguese hospital sewage (2001)	Antimicrobial susceptibility testing	Freitas *et al*., 2009
Listeria monocytogenes FFUP 35	Faculty of Pharmacy of Porto University Culture collection (FFUPCC)	Antimicrobial susceptibility testing	
Gram negative			
Acinetobacter baumanni ATCC 19606	American Type Culture Collection (ATCC)	Antimicrobial susceptibility testing	Reference Strain
Escherichia coli ATCC 25922	American Type Culture Collection (ATCC)	Antimicrobial susceptibility testing	Reference Strain
Pseudomonas aeruginosa ATCC 27853	American Type Culture Collection (ATCC)	Antimicrobial susceptibility testing	Reference Strain
Salmonella Typhymurium F154/22	Food – Portugal (2004)	Antimicrobial susceptibility testing	Antunes *et al*., 2006
Salmonella Enteretidis 62/02	Human – Portugal (2002) - INSA Culture Collection	Antimicrobial susceptibility testing	Antunes *et al*., 2006

Table A3. Oligonucleotides used for the characterization of *Bacillus* isolates.

Description/Gene	Primer name	Oligonucleotide sequence (5´to 3´)	Amplicon size (bp)	Annealing temperature (ºC)	Reference
Identification of *Bacillus* species					
16S rRNA	SEQ A	AGAGTTTGATCHTGGYTYAGA	1500	55	Héritier *et al*., 2003
	SEQ B	ACGYTACCTTGTTACGACTTC			
β-subunit of DNA gyrase (*gyrB*)	UP-1S	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	1400	60	Yamamoto and Harayama, 1995
	UP-2Sr	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT			
β-subunit of RNA polymerase (*rpoB*)	$rpoB_{1\_fw}$	CAGAAGCTACGCACGCATAA	775	56	Branquinho *et al.*, 2014a
	$rpoB_{1\_rv}$	GCGTCCAACATTTGCTAGGT			
	$rpob_{2\_fw}$	CAACACGCTGGAAAAAGACA	1028	56	Branquinho *et al.*, 2014a
	$rpoB_{2\_rv}$	TTCTCGGCGTCACTTTACCT			
	$rpoB_{3\_fw}$	CACCAGAGGGTCCAAACATT	915	56	Branquinho *et al.*, 2014a
	$rpoB_{3\_rv}$	GGTTGCGTAAAGCATCTTCC			
	$rpoB_{4\_fw}$	CATGAGTGAGCGCCTTGTAA	1255	56	Branquinho *et al.*, 2014a
	$rpoB_{4\_rv}$	CGTCTGCTTTCTTCGTTTCC			
PCR-based non-ribosomal lipopeptide biosynthetic genes detection					
Surfactin/Lichenysin family					
srf/lch	As1-F	CGCGGMTACCGVATYGAGC	419/422/425/431	43	Tapi *et al*., 2010
	Ts2-R	ATBCCTTTBTWDGAATGTCCGCC			
Surfactin					
sfp	sfp-f	ATGAAGATTTACGGAATTTA	675	46	Hsieh *et al*., 2004
	sfp-r	TTATAAAAGCTCTTCGTACG			
Lichenysin					

*lchA*A	LicA-f LicA-r	GTGCCTGATGTAACGAATG CACTTCCTGCCATATACC	735	60	Nieminen *et al*., 2007
*lchA*B	LicB2-f LicB2-r	TGATCAGCCGGCCGTTGTCT GGCGAATTGTCCGATCATGTCC	904	60	Nieminen *et al*., 2007
*lchA*C	LicC-f LicC-r	GCCTATCTGCCGATTGAC TATATGCATCCGGCACCA	1195	57	Nieminen *et al*., 2007
Fengycin/Plipastation family					
Fengycin					
fen	Af2-F Tf1-R	GAATAYMTCGGMCGTMTKGA GCTTTWADKGAATSBCCGCC	443/452	45	Tapi *et al*., 2010
*fen*A	FENA1F FENA1R	GACAGTGCTGCCTGATGAAA GTCGGTGCATGAAATGTACG	964	62	Athukorala *et al*., 2009
*fen*B	FENB2F FENB2R	CAAGATATGCTGGACGCTGA ACACGACATTGCGATTGGTA	964	62	Athukorala *et al*., 2009
*fen*D	FEND1F FEND1R	TTTGGCAGCAGGAGAAGTTT GCTGTCCGTTCTGCTTTTTC	964	62	Athukorala *et al*., 2009
Pliplastatin					
pps	Ap1-F Atp1-R	AGMCAGCKSGCMASATCMCC GCKATWWTGAARRCCGGCGG	893/959/929	58	Tapi *et al*., 2010
Iturin family					
Iturin					
lpa-14	lpa-14f lpa-14r	ATGAAAATTTACGGAGTATA TTATAACAGCTCTTCATACG	675	50	Hsieh *et al*., 2008
*itu*D	ituD-f ituD-r	ATGAACAATCTTGCCTTT TTA TTATTTTAAAATCCGCAATT	1203	50	Hsieh *et al*., 2008

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PCR-based virulence detection					
Emetic toxins					
cesA	CER1_Fw EMT1_Rv	ATCATAAAGGACAAGA AAGATCAACCCAACTG	188	52	Ehling-Schulz *et al*., 2004
cesB	EM1_Fw EM1_Rv	GACAAGAGAATACAAT GCAGCCTTCCCACAGT	635	60	Ehling-Schulz *et al*., 2004
Cytotoxins					
cytK	FC_Fw RC_Rv	GTAACTTTCATTTGATGATC GAATACATAAATAATTGGT	505	50	Ehling-Schulz *et al*., 2004
hblC	L2_Fw L2_Rv	AGAAACTCAACAAGAAAACATGG TTGCGCAGTTGCCACATTAG	444	50	Ehling-Schulz *et al*., 2004
nheA	0045_Fw 4091_Rv	GTTAGGATCACAATCACCGC CCATATGCATTTGTAAAATCTG	264	50	Ehling-Schulz *et al*., 2004
nheB	517_Fw 8368_Rv	CGGTTCATCTGTTGCGACAGC GATCCCATTGTGTACCATTGG	335	50	Ehling-Schulz *et al*., 2004
nheC	4924_Fw 1141_Rv	GCGATTGATCAAAAGGATAG CGACTTCTGCTTGTGCTCCTG	411	50	Ehling-Schulz *et al*., 2004
PCR-based resistance genes detection					
Tetracycline					
tet M	tetM-F tetM-R	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	657	53	Aarestrup *et al*., 2000.
tet L	tetL-F tetL-R	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG	475	53	Aarestrup *et al*., 2000.
tet S	tetS-F tetS-R	TGGAACGCCAGAGAGGTATT ACATAGACAAGCCGTTGACC	661	56	Aarestrup *et al*., 2000.

tet K	tetK-F tetK-R	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	718	56	Aarestrup *et al*., 2000.
tet O	tetO-F tetO-R	CAATATCACCAGAGCAGGCT GATGGCATACAGGCACAGAC	634	53	Aarestrup *et al*., 2000.
Macrolides					
ermA	ermA1-F ermA2-R	CTTCGATAGTTTATTAATATTAGT TCTAAAAAGCATGTAAAAGAA	645	50	Sutcliffe *et al*., 1996
ermB	ermB1-F ermB2-R	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	402	50	Sutcliffe *et al*., 1996
ermC	ermC1-F ermC2-R	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	642	50	Sutcliffe *et al*., 1996
Glycopeptides					
vanA	vanA-F vanA-R	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	54	Dutka-Malen *et al*., 1995
vanB	vanB-F vanB-R	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	54	Dutka-Malen *et al*., 1995
Aminoglicosydes					
aph(2")-Ib	aph(2")-IbF aph(2")-IbR	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCC TT	867	52	Vakulenko *et al*., 2003
aph(2")-Id	aph(2")-IdF aph(2")-IdR	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	641	52	Vakulenko *et al*., 2003
aph(2")-Ic	aph(2")-IcF aph(2")-IcR	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG	444	52	Vakulenko *et al*., 2003
aph(3")-IIIa	aph(3")-IIIaF aph(3")-IIIaR	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	52	Vakulenko *et al*., 2003

aac(6')-Ie-aph(2'')-Ia	aac(6')-Ie-aph(2'')-IaF- aac(6')-Ie-aph(2'')-IaR	CATTATACAGAGCCTTGGGAAGATG GTCTTAAAAAACTGGCAATATCTCGTT	778	52	Vakulenko *et al*., 2003
Chloramphenicol					
fexA	fexA-F fexA-R	GTACTTGTAGGTGCAATTACGGCTGA CGCATCTGAGTAGGACATAGCGTC	1272	58	Kehrenberg and Schwarz 2006
Florfenicol					
floR	floR_F floR_R	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	868	55	Guerra *et al*., 2004
Phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A					
cfr	cfr-fw cfr-rv	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	746	48	Kehrenberg and Schwarz 2006

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